

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 270 799 B1

(12)

EUROPEAN PATENT SPECIFICATION

- (45) Date of publication of patent specification: **21.09.94** (51) Int. Cl.⁵: **A61K 37/66, A61K 47/08, A61K 47/10, A61K 47/14**
- (21) Application number: **87115693.1**
- (22) Date of filing: **26.10.87**

(54) **Pharmaceutical compositions of recombinant beta-Interferon and formulation processes.**

- (30) Priority: **27.10.86 US 923423**
- (43) Date of publication of application:
15.06.88 Bulletin 88/24
- (45) Publication of the grant of the patent:
21.09.94 Bulletin 94/38
- (84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE
- (56) References cited:
EP-A- 0 107 498
EP-A- 0 150 067
EP-A- 0 152 345
EP-A- 0 215 658
US-A- 4 606 917
- CHEMICAL ABSTRACTS, vol. 106, 06 April 1987, Columbus, OH (US); p. 373, no. 107907k&NUM;**
- CHEMICAL ABSTRACTS, vol. 94, 1981, Columbus, OH (US); p. 312, no. 20423j&NUM;**
- (73) Proprietor: **CETUS ONCOLOGY CORPORATION**
1400 Fifty-Third Street

Emeryville California 94608 (US)

- (72) Inventor: **Shaked, Ze'ev**
1038 Sierra Street
Berkeley, CA 94707 (US)
Inventor: **Stewart, Tracy**
2376 Bryant Street
San Francisco, CA 94110 (US)
Inventor: **Thomson, Jody**
811 Talbot Avenue
Albany, CA 94706 (US)
Inventor: **Thomson, James William**
811 Talbot Avenue
Albany, CA 94706 (US)
Inventor: **Taforo, Terrance**
379A, Somerset Road
Oakland, CA 94611 (US)
Inventor: **Hershenson, Susan**
1275 Eighth Avenue, No. 103
San Francisco, CA 94122 (US)
- (74) Representative: **VOSSIUS & PARTNER**
Postfach 86 07 67
D-81634 München (DE)

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

Description

This invention is in the field of biochemical engineering. More particularly, the invention concerns improved pharmaceutical compositions of biologically active recombinant beta-interferon (IFN- β) protein which is suitable for therapeutic administration to humans. Further, the invention concerns an improved process for preparing and formulating such IFN- β compositions.

Naturally occurring interferons (IFNs) are species-specific proteins, often glycoproteins, produced by various cells upon induction with viruses, double stranded RNAs, other polynucleotides, antigens and mitogens. Interferons exhibit multiple biological activities such as antiviral, antiproliferative, immunomodulatory and anticellular functions. At least three distinct types of human interferons have been identified and characterized in terms of their anti-viral, anti-growth and activation of natural killer cell (NK) activities. They are produced by leukocytes, lymphocytes, fibroblasts and the immune system and are classified as α , β and γ interferons. These are reported to be different proteins coded for by distinct structural genes.

Native human β -interferon (HuIFN- β) is generally produced by superinducing human fibroblast cultures with poly-IC (polyriboinosinic acid and polyribocytidylic acid) and isolating and purifying the HuIFN- β thus produced by chromatographic and electrophoretic techniques. Proteins or polypeptides which exhibit native β -interferon may also be produced using recombinant DNA technology by extracting poly-A-rich 12S messenger RNA from virally induced human cells, synthesizing double-stranded c-DNA using the m-RNA as a template, introducing the c-DNA into an appropriate cloning vector, transforming suitable microorganisms with the vector, harvesting the bacteria and extracting the HuIFN- β therefrom. Nagola, S. et al., *Nature*, 284:316 (1980); Goeddel, D.V. et al., *Nature*, 287:411 (1980); Yelverton, E. et al., *Nuc. Acid Res.*, 9:731 (1981); Streuli, M. et al., *Proc. Nat'l. Acad. Sci. (U.S.)*, 78:2848 (1981); European Pat. Application Numbers 28033, published May 6, 1981; 321134, published July 15, 1981; 34307 published August 26, 1981; and Belgian Patent 837397, issued July 1, 1981 describe various currently used methods for the production of β -interferon employing recombinant DNA techniques. The expressed proteins or polypeptides have been purified and tested and have been found to exhibit properties similar to those of native IFNs. Bacterially produced IFNs thus appear to have potential therapeutic use as antiviral and anti-tumor agents and the production of IFNs by such bacterial fermentations is expected to yield sufficiently large quantities of IFN at a relatively low cost of clinical testing.

Further, HuIFN- β genes have been altered by, for example, oligonucleotide-directed mutagenesis to produce IFN- β protein analogs thereof, such as the human recombinant cysteine-depleted or cysteine-replaced interferon- β analogs (muteins) disclosed in U.S. Patent No. 4,588,585 issued May 13, 1986 to Mark et al. Specifically disclosed in that patent is the recombinant IFN- β mutein wherein the cysteine at position 17 is replaced by a neutral amino acid such as serine. The latter IFN- β mutein is IFN- β_{Ser17} .

Procedures for recovering and purifying bacterially produced IFNs are described in U.S. Patent Nos. 4,450,103; 4,315,852; 4,343,735; and 4,343,736; and Derynck et al., *Nature* (1980) 287:193-197 and Scandella and Kornberg, *Biochemistry*, 10:4447 (1971). Generally with these methods the IFN is not produced in a sufficiently pure form and in sufficiently large quantities for clinical and therapeutic purposes and the resulting IFN preparations produced by recombinant DNA techniques have residual amounts of chemicals, such as sodium dodecyl sulfate (SDS) and other surfactants or precipitants used in the extraction and purification steps.

E. coli expressed recombinant IFN- β and analogs thereof are insoluble in solutions which are at a pH range of 6 to 9. Therefore, various processes and additives have been devised to solubilize these proteins. Several methods currently available for the preparation, recovery and purification of bacterially produced proteins are listed immediately below.

U.S. Patent No. 4,315,852 to Leibowitz et al. describes a method for the acid extraction of leukocyte interferon from bacterial cells and neutralization of the extractant to obtain the interferon.

U.S. Patent No. 4,343,735 to Menge et al. teaches a process for the purification of interferon by partitioning it in an aqueous multi-phase system in the presence of ion exchangers which are soluble in the system and are derivatives of polyethers.

U.S. Patent No. 4,343,736 to Uemura et al. discloses a method for recovering interferon by absorption of water-insolubilized heparin and then eluting the interferon with an aqueous solution of an inorganic salt and chondroitin sulfate.

U.S. Patent No. 4,289,689 to Friesen et al. discloses how to recover and purify human native β -interferon by use of affinity chromatography and high pressure liquid chromatography.

U.S. Patent No. 4,364,863 to Leibowitz et al. describes a method of extracting fibroblast interferon from bacteria using a low pH followed by a high pH extraction procedure.

U.S. Patent No. 4,450,103 to Konrad et al. discloses solubilizing the protein in an aqueous medium with an appropriate solubilizing agent, extracting the protein from the aqueous medium with 2-butanol or 2-methyl-2-butanol, and precipitating the protein from the alcohol phase.

U.S. Patent No. 4,530,787 to Shaked et al. describes a process for oxidizing recombinant proteins such as IFN- β selectively and stoichiometrically using *o*-iodosobenzoic acid to ensure that the protein will be functionally equivalent to its native counterpart.

Many heterologous proteins are precipitated intracellularly in the form of refractile or inclusion bodies which appear as bright spots visible within the enclosure of the cell under a phase contrast microscope at magnifications down to 1000 fold. See e.g., Miller et al., *Science* (1982) 215:687-690; Cheng, *Biochem. Biophys. Res. Comm.*, (1983) 111:104-111; Becker et al., *Biotech. Adv.* (1983) 1:247-261; Kleid et al., ch. 25 in *Developments in Industrial Microbiology*, Vol. 25, p. 317-325 (Society for Industrial Microbiology, Arlington, VA, 1984); Marston et al., *Bio/Technology* (September, 1984), pp. 800-804.

Purification and activity assurance of precipitated heterologous proteins is also described by U.S. Patent Nos. 4,511,502; 4,511,503; 4,512,922; 4,599,127 and 4,518,526; and EP 114,506.

EP 206,828 discloses improved methods for recovering and purifying refractile bodies.

Wang et al., *J. Parenteral. Drug Assoc.*, 34, 452-462 (1980) provides a review of excipients and pHs for parenteral products used in the United States. A list of solubilizing agents such as detergents and lipids in use for various drugs is provided in Table I thereof and under section II entitled "Solubilizers, Wetting Agents or Emulsifiers" of that table, polyethylene glycol 300, polysorbate 20, 40 and 80, and propylene glycol among others are listed for a variety of pharmaceuticals. Below are referenced some examples of interferon and related formulations.

U.S. Patent No. 4,647,454 to Cymalista et al. discloses a method of stabilizing human fibroblast interferon with polyvinyl pyrrolidone.

U.S. Patent No. 4,460,574 to Yabrov discloses a pharmaceutical composition comprising native human α - and β -interferons used for rectal or urogenital treatment of human interferon-sensitive diseases.

U.S. Patent No. 4,462,940 to Hanisch et al. ('940 patent) discloses a process for formulating interferon by mixing the interferon and a protein stabilizer, such as normal serum albumin, at a pH of about 10.5 to 12.5 for 5 minutes and then adjusting the pH to 7.5 to obtain a soluble mixture. The '940 patent to Hanisch et al. further describes a method for recovering and purifying a lipophilic protein such as human recombinant interferon- β or IL-2 which comprises solubilizing the protein into an aqueous medium with a suitable solubilizing agent, such as sodium dodecyl sulfate (SDS), extracting the solubilized protein with an aliphatic alcohol, precipitating the protein from the alcohol phase with an aqueous buffer, and diafiltering the protein at a pH of about 10.5 to 12.5 against water or against mixtures of water and aliphatic alcohols adjusted to a pH of about 10.5 to 12.5, to remove the solubilizing agent (SDS) or reduce its concentration.

One aspect of the instant invention concerns a process alternative to the method described above and claimed in U.S. Patent No. 4,462,940. Said process alternative avoids the high pH range (pH 10.5 to 12.5) required to remove certain solubilizing agents such as SDS during diafiltration or desalting, preferably desalting.

EP 215,658 outlines a high pH and a low pH process for recovering and purifying lipophilic recombinant proteins, such as human IFN- β from host strains to yield a protein preparation which may be formulated into a stable pharmaceutical composition. Said composition carrying a therapeutically effective amount of the biologically active lipophilic protein dissolved in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 6.8 to 7.8 also contains a stabilizer for the protein, such as human serum albumin, human serum albumin and dextrose, or human plasma protein fraction.

EP 217,645 discloses pharmaceutical compositions containing IFN- β or interleukin-2 dissolved in a stable carrier medium at pH 7.0 to 8.0 stabilized with sodium laurate.

WO 87/00056 discloses pharmaceutical compositions wherein recombinant IFN- β , IL-2 or an immunotoxin is dissolved in an aqueous carrier medium at pH 6 to 8 without the presence of a solubilizing agent. The protein is solubilized by selectively conjugating it via a coupling agent to a water-soluble polymer selected from polyethylene glycol homopolymers or polyoxyethylated polyols.

Japanese Laid-Open Patent Application (Kokai) No. 59-10524 (published January 20, 1984) entitled "An Interferon Composition and a Method of Manufacturing the Same" discloses a micelle solution for rectal administration prepared by mixing (a) an unsaturated fatty acid, (b) a polyoxyethylene fatty acid ester, alkyl polyoxyethylene ether or sucrose fatty acid ester, (c) water and (d) interferon.

EP 135,171 discloses pseudomonophase, microemulsion compositions for topical application of interferons, preferably leukocyte interferon A. The compositions comprise a therapeutically effective amount of interferon, 30-70% by volume of a surface active agent having a hydrophilic-lipophilic balance (HLB) of from 12-15 and dual solubility in water/oil; 5-45% of a vegetable oil; and 5-45% water. The surface active agents

disclosed there in are polyethylene glycol derivatives of castor oil composed on average of 25-36 moles of ethylene oxide per mole of castor oil. Such an oil-based microemulsion is not stable and subject to phase separation.

U.S. Patent No. 4,507,281 discloses compositions comprising about 10^4 to 10^6 I.U. of human leukocyte interferon, about 1% to 5% by weight of a non-ionic surface active agent having at least one ether or amide linkage, and a physiologically acceptable carrier. Such compositions are topically administered to treat herpes simplex infections wherein a therapeutic effect is attributed to such non-ionic surfactants in their ability to dissolve the lipid-containing envelope of the herpes simplex virus. The preferred non-ionic surfactants referred to therein include: nonylphenoxypolyethoxy ethanol (trade name Nonoxynol-9); p-diisobutylphenoxypolyethoxy ethanol (trade name Triton X-100); polyoxyethylene (10) oleyl ether (trade name Brij-97); and onyx-ol (trade name Onyx-ol 345). Such pharmaceutical compositions are preferably administered in lotion, cream, oil, or emulsion formulations. See also EP 77,063 which is a foreign equivalent thereof.

There remains a need in the art for formulations of biologically active, recombinant beta-interferons that are pure enough for clinical administration but substantially or totally free of residual strong detergents, such as SDS, used in the extraction and purification processes. Further, there is a need for formulations that provide alternatives to those containing non-IFN- β protein, such as those containing albumin.

Further, alternative formulation processes for such biologically active, recombinant beta-interferons which avoid very high pH ranges are desirable.

The present invention provides for stable pharmaceutical compositions of matter suitable for parenteral administration to animals or humans comprising a therapeutically effective amount of a recombinant interferon- β protein dissolved in an inert carrier medium comprising as a solubilizer/stabilizer an effective amount of one or more biocompatible non-ionic polymeric detergents. Such compositions can further comprise an additional solubilizing or stabilizing agent, for example, sodium dodecyl sulfate (SDS) and glycerol.

The use of glycerol in combination with one or more non-ionic detergents of this invention allows for an order of magnitude decrease in the concentration range of the non-ionic detergents required to solubilize/stabilize the recombinant IFN- β .

The formulations of this invention can be in liquid form or lyophilized.

Another aspect of the invention is methods of screening for one or more biocompatible non-ionic polymeric detergents or for combinations of one or more of said detergents with another solubilizing or stabilizing agent capable of solubilizing and stabilizing pharmaceutical compositions of recombinant interferon- β (IFN- β), comprising the steps of:

- (a) passing extracted, purified recombinant IFN- β in sodium dodecyl sulfate (SDS) on a desalting column equilibrated in sodium laurate in an elution buffer at pH 9.0-10.0 to form an eluate;
- (b) lowering the pH of the eluate to pH 2-3.3;
- (c) centrifuging and filtering the eluate to remove the precipitated sodium laurate;
- (d) adding to the filtrate an effective amount of one or more of said detergents or said combination of one or more of said detergents with another solubilizing or stabilizing agent;
- (e) adjusting the pH of the filtrate to 3.5 to 9.5; and
- (f) allowing said filtrate to stand for 24 hours at pH 3.5 to 9.5.

The invention further provides for methods of preparing stable, pharmaceutical compositions of recombinant interferon- β (IFN- β) protein wherein one such method comprises the steps of:

- (a) extracting the recombinant IFN- β from the disruptate of a host organism transformed to produce the protein;
- (b) purifying the recombinant IFN- β using as the last purification step a desalting step at a pH range of 8.5 to 10 employing an elution buffer containing a fatty acid salt having a carbon chain containing from about 10 to about 12 carbons to form a desalted pool;
- (c) lowering the pH of the desalted pool to a pH of 2 to 4 thereby precipitating the fatty acid salt;
- (d) removing the precipitated salt from the pool by centrifugation and filtration;
- (e) adding to the desalted pool an effective amount of one or more non-ionic biocompatible polymeric detergents, or a combination of one or more biocompatible non-ionic polymeric detergents and another solubilizing or stabilizing agent, in effective amounts to stabilize and solubilize the recombinant IFN- β ;
- (f) adjusting the pH of the pool to a range of between 4.0 and 8.0;
- (g) adding an effective amount of a bulking/stabilizing agent to the pool; and
- (h) lyophilizing the formulation; and

wherein another such method comprises the steps of:

- (a') isolating refractile bodies containing the recombinant IFN- β from a host organism transformed to produce said recombinant IFN- β ;
- (b') solubilizing said r fractile bodies by employing sodium laurate;
- (c') extracting and purifying said recombinant IFN- β from the solubilized r fractile material employing sodium laurate as the primary solubilizing agent;
- (d') lowering the pH of the purified recombinant IFN- β to a pH from 2 to 4;
- (e') centrifuging and filtering the purified recombinant IFN- β solution to remove the sodium laurate precipitate to create a recombinant IFN- β pool;
- (f') desalting the recombinant IFN- β pool at a pH from 2 to 4;
- (g') adding to the recombinant IFN- β protein solution an effective amount of one or more non-ionic biocompatible polymeric detergents, or a combination of a non-ionic biocompatible polymeric detergent and another solubilizing or stabilizing agent to stabilize and solubilize the recombinant IFN- β ;
- (h') adjusting the pH of the recombinant IFN- β solution to a pH range of 3.5 to 9.5;
- (i') adding to the solution an effective amount of a bulking/stabilizing agent; and
- (j') lyophilizing the formulation.

The invention still further concerns a method of extracting recombinant interferon- β (IFN- β) from a bacterial host transformed to produce it and then purifying and formulating said recombinant IFN- β comprising the steps of:

- (a) growing the transformed bacterial hosts in an appropriate fermentation medium;
- (b) concentrating the bacterial host in the fermentation medium;
- (c) disrupting the cell wall and cell membrane of the bacterial host;
- (d) removing greater than 99% by weight of the salts from said disruptate by diafiltration or centrifugation;
- (e) redispersing the desalted disruptate;
- (f) adding a material to the disruptate to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate;
- (g) separating the refractile material from the cellular debris by high-speed centrifugation;
- (h) solubilizing the refractile material in an aqueous buffer containing a reducing agent;
- (i) extracting the solubilized refractile material with 2-butanol or 2-methyl-2-butanol;
- (j) isolating said refractile material from the extractant;
- (k) solubilizing the resulting recombinant IFN- β particle pellet with an aqueous solution of sodium dodecyl sulfate at a recombinant IFN- β to sodium dodecyl sulfate ratio of 1:3 to form a solution;
- (l) adjusting the pH of the solution to 9.5 and reducing the solubilized recombinant IFN- β with dithiothreitol;
- (m) purifying the reduced recombinant IFN- β by chromatography;
- (n) oxidizing the recombinant IFN- β from step (m);
- (o) further purifying the oxidized recombinant IFN- β by gel chromatography and collecting the eluate containing the purified recombinant IFN- β ;
- (p) desalting the purified recombinant IFN- β eluate in a desalting column equilibrated and run in sodium laurate at pH 9.0 to 9.8;
- (q) lowering the pH of the eluate to pH 3.0;
- (r) centrifuging and filtering the solution to remove the precipitate;
- (s) adding to the filtrate an effective amount of one or more non-ionic biocompatible polymeric detergents or a combination of one or more of said detergents and another solubilizing or stabilizing agent to solubilize and stabilize the recombinant IFN- β ;
- (t) adjusting the pH of the solution to 4.0 to 9.5;
- (u) adding an appropriate bulking/stabilizing agent in a concentration of from about 0.25% to about 10%; and
- (v) lyophilizing the recombinant IFN- β solution.

Figures 1A and 1B sequentially illustrate the steps of a preferred embodiment of the instant invention for extracting, purifying and formulating microbially produced recombinant IFN- β . In this flow chart sodium laurate is used as the primary solubilizing agent in the extraction and purification process. Macol® LA-12 (0.15%) is the formulating agent (solubilizer/stabilizer in the formulation) and dextrose (5%) is the bulking/stabilizing agent.

Figures 2A and 2B outline the steps of another preferred embodiment of the invention wherein SDS is employed as the primary solubilizing agent during extraction and purification, and sodium laurate is employed as a transfer component during desalting on a G-25 column to remove the SDS. Plurafac® C-17 (0.1%) is the formulating agent (solubilizer/stabilizer in the formulation) and dextrose (5%) is the bulk-

ing/stabilizing agent.

Figures 3-6 graphically illustrate linear non-isothermal stability (LNS) study results comparing representative formulations of this invention with sodium laurate and human serum albumin (HSA) IFN- β formulations. The HSA formulations are indicated in these figures as normal, high and low dose.

This invention provides for highly stable pharmaceutical compositions of matter suitable for parenteral administration to animals or humans comprising a therapeutically effective amount of a recombinant interferon- β protein dissolved in an inert carrier medium comprising one or more biocompatible non-ionic polymeric detergents or the combination of one or more non-ionic biocompatible polymeric detergents with an additional solubilizing/stabilizing agent.

The term "recombinant β -interferon," designated as IFN- β , preferably human recombinant IFN- β , refers to fibroblast interferon having biological activity comparable to native IFN- β , prepared by recombinant DNA techniques as described in the art. In general, the gene coding for interferon is excised from its native plasmid and inserted into a cloning vector to be cloned and then into an expression vector, which is used to transform a host organism, preferably a microorganism, and most preferably *E. coli*. The host organism expresses the foreign interferon gene under certain conditions to produce IFN- β . More preferably, the recombinant IFN- β is a mutein as described in U.S. Patent No. 4,588,585, in which the cysteine normally occurring at position 17 of the wild-type or native molecule has been replaced by a neutral amino acid, such as, serine or alanine. Most preferably, the recombinant IFN- β mutein is IFN- β_{ser17} .

The precise chemical structure of the recombinant IFN- β protein will depend on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular recombinant IFN- β protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their bioactivity when placed in suitable environmental conditions are included in the definition of recombinant IFN- β proteins herein. Further, the primary amino acid sequence of the protein may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups more commonly by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced in vitro. In any event, such modifications are included in the definition of recombinant IFN- β protein herein so long as the biological activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays.

Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy biological activity do not remove the protein sequence from the definition.

The pharmaceutical compositions of this invention provide a means of maintaining recombinant IFN- β in soluble form and thereby stabilizing it in pH ranges wherein recombinant IFN- β is not readily soluble, by use of one or more solubilizer/stabilizers of this invention. Recombinant IFN- β unlike native IFN- β , is considered to be lipophilic and hydrophobic, that is, insoluble or not readily soluble in water or an aqueous medium under ambient conditions of room temperature and atmospheric pressure at a pH of between about 5 and about 8. The most difficult recombinant IFN- β solubility problems are those that nonglycosylated recombinant IFN- β produced by a transformed bacteria most, most notably *E. coli*. Therefore, the pharmaceutical compositions of this invention are especially useful in solubilizing/stabilizing nonglycosylated recombinant IFN- β produced in *E. coli*, and it is preferred that the recombinant IFN- β of the pharmaceutical compositions of this invention be of such origin.

As used herein, the term "physiological pH" refers to a pH which is pharmaceutically acceptable to mammals. Neutral pH is herein considered to be a pH in the range of 6.0 to 8.5, preferably about 6.0 to 8.0.

As used herein, the term "stabilizer/solubilizer" as applied to the recombinant IFN- β formulations refers to essentially non-toxic and non-immunogenic compositions which alone or in combination act not only to stabilize the recombinant IFN- β against denaturation and loss of biological activity, but also to solubilize the lipophilic protein in an aqueous medium so that the pharmaceutical formulation constitutes an aqueous solution of recombinant IFN- β protein at pH 3.5 to 9.5, preferably 4 to 8, more preferably 5.5 to 6.5, and still more preferably pH 6, from which the protein will not precipitate. The stabilizer/solubilizer compositions of the invention are one or more biocompatible, non-ionic polymeric detergents, or combinations of one or more biocompatible, non-ionic polymeric detergents with another solubilizing and/or stabilizing agent.

The term "primary solubilizing agent" is defined herein to mean a solubilizing agent, preferably a detergent and/or chaotrope, which is used to solubilize the recombinant IFN- β from the refractile bodies in the abbreviated or expanded front-end processes of purifying IFN- β described *infra*. The primary solubilizing agent is preferably then relied upon to maintain the recombinant IFN- β in solution throughout the purification process up to its removal, preferably by desalting, before formulating the purified recombinant

IFN- β with the non-ionic detergents according to this invention. For example, in the purification scheme illustrated by Figures 1A and 1B, sodium laurate is the primary solubilizing agent, whereas, sodium dodecyl sulfate (SDS) is the primary solubilizing agent in the process illustrated by Figures 2A and 2B.

As used herein the term "transformed" in describing host microorganism cell cultures denotes a microorganism that has been genetically engineered to produce IFN- β that possesses biological activity comparable to native IFN- β . Bacteria are preferred microorganisms for producing IFN- β . *E. coli* is particularly preferred.

"Chaotropic environment" refers to an environment in which proteins are denatured or changed from their ordinary conformations. Chaotropic environments may be engendered by the presence of suitable concentrations of chaotropic agents, as described below, or may be the result of heat or pH alterations. The resultant environments are capable of disrupting hydrogen bonding in the protein and altering the thermodynamics of the surroundings in such a way that alternate three-dimensional conformations are preferred in the chaotropic environment to those found in more physiologically compatible environments.

The term "chaotropic agent" refers to a compound or compounds which, in aqueous solution and in a suitable concentration, engender a chaotropic environment and are capable of denaturing IFN- β . Guanidine salts (e.g., the hydrochloride) and alkali metal thiocyanates (e.g., sodium thiocyanate) and urea at concentrations in the range of 4 to 9M, preferably 6 to 9M, are examples of chaotropic environments that will dissolve and denature recombinant IFN- β .

"Reducing conditions" are those required to place or maintain the recombinant IFN- β in reduced form with respect to the cysteine residues. These conditions can most simply be provided by use of a suitable reducing agent (especially a thiol-containing reducing agent), or if the recombinant IFN- β is already reduced (e.g., in the cellular environment), exclusion of air and oxidation catalysts or reagents may suffice.

The biocompatible non-ionic polymeric detergents are essentially non-toxic surface active agents used in the food, pharmaceutical, and cosmetic industries and have molecular weights in the range of 100 to 250,000, and more preferably from 180 to 200,000. The term "detergent" herein is broadly defined to include compounds or mixtures of compounds known to those skilled in the art as detergents, emulsifiers, solubilizers, surfactants and/or surface-active agents.

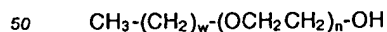
The biocompatible non-ionic polymeric detergents used as solubilizer/stabilizers in the formulations of this invention are characterized by their ability either alone or in combination with another non-ionic detergent or with another type of solubilizing/stabilizing agent, preferably sodium dodecyl sulfate (SDS) or glycerol, to solubilize recombinant IFN- β at a pH range of from 3.5 to 9.5, more preferably from 5 to 8.

The amphiphilic nature of non-ionic detergents is often expressed in terms of the balance between the hydrophobic and hydrophilic portions of the molecule. An empirical scale of hydrophile-lipophile balance numbers (HLB) has been devised. An HLB number is a value extending from 1 to approximately 50, which indicates the extent of hydrophilicity or lipophilicity of a surface-active agent. The more hydrophilic surfactants have high HLB numbers (in excess of 10), whereas surfactants with HLB numbers from 1 to 10 are considered to be lipophilic.

Preferable biocompatible non-ionic polymeric detergents in the formulations of this invention have hydrophile-lipophile balance (HLB) numbers in the range of from 10 to 40, preferably from 15 to 35, and more preferably from 12 to 30. Further such biocompatible non-ionic polymeric detergents are preferably selected from the group comprising ethoxylated fatty alcohol ethers and lauryl ether, octylphenoxy polyethoxy ethanol compounds, modified oxyethylated and/or oxypropylated straight-chain alcohols, polyethylene glycol monooleate compounds, polyoxyethylene sorbitan fatty acid esters, phenolic fatty alcohol ethers and block copolymers of propylene oxide and ethylene oxide (polyoxypropylene and polyoxyethylene condensates).

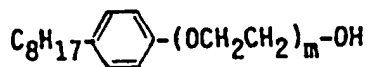
Further, preferably the biocompatible non-ionic polymeric detergents employed as stabilizer/solubilizers in the formulations of the instant invention include:

1. a mixture of ethoxylated fatty alcohol ethers and lauryl ether having the formula:



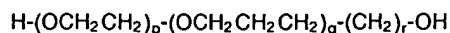
wherein n is a distribution from 1 to 30, and preferably centering on n = 12, and w is a distribution from 9 to 17, containing a significant proportion of w = 11; more preferably wherein said mixture of ethoxylated fatty alcohol ethers and lauryl ether are known as polyoxyethylene 12 lauryl ether or polyoxyethylene 12 lauryl alcohol; still more preferably said mixture is selected from the group of commercially available detergents known as Trycol® LAL-12, Macol® LA-12 and Siponic® L-12, most preferably Macol® LA-12;

2. an octylphenoxy polyethoxy ethanol compound having the formula:



wherein m is an integer between 5 and 60, more preferably wherein m is an integer between 20 and 50, still more preferably wherein m is an integer between 30 and 40, further more preferably wherein m is either 30 or 40; most preferably wherein m is 30, the detergent is Triton® X305, and wherein m is 40, the detergent is Triton® X405;

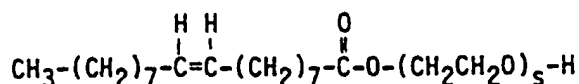
3. a mixture of modified oxyethylated and/or oxypropylated straight-chain alcohols having the formulas:



wherein p is an integer between 1 and 10;

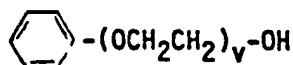
wherein q is zero or an integer between 1 and 10; and wherein r is an integer between 6 and 14; more preferably, wherein p is an integer between 1 and 5, q is zero or an integer between 1 and 5; and r is an integer between 10 and 12, most preferably when said detergent is Plurafac® C-17;

4. a polyethylene glycol monooleate compound having the formula:



wherein s is an integer between 1 and 10, and wherein the molecular weight of said compound is in the range of from 300 to 750; more preferably, wherein s is an integer between 2 and 6, and wherein the molecular weight of said compound is from 350 to 550; still more preferably, wherein s is 3 and the molecular weight of said compound is 400; and most preferably, wherein said detergent is Nopalcol® 4-0;

5. a phenolic fatty alcohol ether having the formula:



wherein v is an integer between 10 and 200, more preferably, wherein v is an integer between 50 and 150, and cost preferably, wherein v is 100 and said ether has an HLB of 19; and

6. block copolymers of propylene oxide and ethylene oxide which are Pluronic polyols; more preferably, wherein said detergent is Pluronic® F68.

Trycol® LAL-12 has an HLB of 14. Plurafac® C-17 has an HLB of 16. Nopalcol® 4-0 has an HLB of 12. Durfax® 80 has an HLB of 15.9. Triton® X305 has an HLB of 17.3. Triton® X405 has an HLB of 17.9. Pluronic® F68 has an HLB of 29. Macol® LA-12 and Siponic® L-12 both have HLBs of 14.5.

Laureth 12 detergents are defined herein as non-ionic biocompatible polymeric detergents which are mixtures of ethoxylated fatty alcohol ethers and lauryl ether, having the formula outlined above under 1. Herein the terms Laureth 12, polyoxyethylene 12 lauryl ether and polyoxyethylene 12 lauryl alcohol, POE (12) lauryl ether and POE (12) lauryl alcohol are considered interchangeable. Preferred brand names of such Laureth 12 detergents are Macol® LA-12, Trycol® LAL-12 and Siponic® L-12, wherein Macol® LA-12 is most preferred.

The above-noted biocompatible non-ionic polymeric detergents are all commercially available from companies addressed in the USA as follows:

| | | |
|----|--|--|
| | Durfax® 80 | |
| 5 | SCM Durkee Foods Huntington Bldg. 925 Euclid Avenue Cleveland, OH 44115 | |
| | Plurafac® C-17 | Pluronic® F68 |
| 10 | BASF Wyandotte Corp. 100 Cherry Hill Road Parsippany, N.J. 07054 | BASF Wyandotte Corp. 101 Cherry Hill Road Parsippany, N.J. 07054 |
| | Nopalcol® 4-0 | Siponic® L-12 |
| 15 | Diamond Shamrock Process Chemicals Division 350 Mt. Kemble Avenue Morristown, N.J. 07960-1931 | Alcolac Inc. 3440 Fairfield Road Baltimore, MD 21226 |
| | Triton® X305 and Triton® X405 | Macol® LA-12 |
| 20 | Rohm and Haas Delaware Valley 5000 Richmond Street Philadelphia, PA 19105 | Inc. Mazer Chemicals, Inc. 3938 Porett Drive Gurnee, IL 60031 |
| | Trycol® LAL-12 | |
| 25 | Emery Chemicals P.O. Box 628 Mauldin, S.C. 29662 | |

Further biocompatible non-ionic polymeric detergents having the above-noted parameters can be found in editions of McCutcheon's Emulsifiers & Detergents published by the McCutcheon Division of MC Publishing Co., 175 Rock Road, Glen Rock, NJ (USA). Such non-ionic polymeric detergents can be selected for the formulations of this invention by the screening processes herein outlined. Further, said screening processes can be used to test candidate non-ionic detergents singly or in combination with or without another solubilizing and/or stabilizing agent, preferably, SDS or glycerol.

Suppliers of several of the biocompatible non-ionic polymeric detergents included as solubilizer/stabilizers of this invention reported the results of toxicity studies performed by them as follows:

| | Reported Toxicology |
|----|---|
| 40 | Durfax® 80 Trycol® LAL(12) Nopalcol® 4-0 No toxic effects when given at 1 g/day for 10 months in rhesus monkey. Acute oral LD ₅₀ 2.2 g/kg in rabbit. Acute oral LD ₅₀ is greater than 21.5 ml/kg in rat. Intravenous LD ₅₀ is 1.08 g/kg in mice. |

Example 10, *infra*, confirms the essentially non-toxic nature of the biocompatible, non-ionic polymeric detergents employed as stabilizer/solubilizers in the formulations of this invention.

Specific examples of preferred combinations of non-ionic biocompatible polymeric detergents that are successful solubilizer/stabilizers of a normal dose (0.25 mg/ml) of recombinant IFN- β include the combination of 0.15% polyoxyethylene 12 lauryl ether (preferably, selected from Trycol® LAL-12, Siponic® LA-12 and Macol® LA-12) and 0.05% Nopalcol 4-0; 0.1% Triton X-305/0.05% Nopalcol 4-0; and 0.1% Triton X405/0.05% Nopalcol 4-0.

The formulations of this invention are in liquid form or lyophilized. Considered first herein are liquid formulations.

The liquid formulations are preferably maintained at a temperature range of from -70°C to +10°C. The frozen formulations are preferably maintained at a temperature range of -70°C to -20°C, whereas the stabilized liquid formulations are preferably maintained at a normal refrigeration range, preferably from +2°C to +8°C.

The liquid formulations of this invention comprise:

- 1) recombinantly produced, purified IFN- β ;
- 2) one or more biocompatible non-ionic polymeric detergents or a combination of one or more non-ionic detergents with another solubilizing and/or stabilizing agent; and
- 3) a small amount of buffer that maintains the formulation at a physiologically acceptable pH range.

The liquid formulations allow for the use of glycerol, an alternative solubilizing and/or stabilizing agent that is not a non-ionic detergent but when used in combination with one or more non-ionic detergents reduces the concentration of the non-ionic detergents required to solubilize/stabilize the recombinant IFN- β by an order of magnitude. Without the presence of glycerol, the preferred concentration ranges for the biocompatible polymeric non-ionic detergents in the formulations of this invention as a solubilizer/stabilizer are from 0.005% to 5%, preferably from 0.01% to 3%, and more preferably from 0.05% to 1.5%. (The concentration ranges for the non-ionic detergent solubilizer/stabilizers of this invention are on a volume/volume basis except for the block copolymers of propylene oxide and ethylene oxide, such as the Pluronic polyols, for which the concentration ranges are on a weight/volume basis.) However, when glycerol is present in the liquid formulations of this invention at a concentration range by volume of from 5% to 50%, preferably from 20% to 30% and more preferably 25%, the one or more non-ionic detergents acting in combination with the glycerol need only be at one-tenth of the concentrations stated above, and the preferred concentration ranges for such non-ionic detergents, alone or in combination, are from 0.0005% to 5%, preferably 0.001% to 1%, and more preferably from 0.01% to 0.5%.

The other preferred additional solubilizing and/or stabilizing agents that can be used in combination with one or more non-ionic detergents of this invention are stronger solubilizing and/or stabilizing agents, preferably sodium dodecyl sulfate (SDS), which can be used in both liquid and lyophilized formulations. Only very small amounts of such a stronger solubilizing and/or stabilizing agent is required in combination with one or more non-ionic detergents to prevent the recombinant IFN- β from precipitating from the liquid or lyophilized/reconstituted formulations of this invention, and preferably SDS would be in a concentration range of from 100 to 200 $\mu\text{g}/\text{mg}$ of recombinant IFN- β , more preferably from 150 to 200 $\mu\text{g}/\text{mg}$ of recombinant IFN- β , and still more preferably from 150 to 173 $\mu\text{g}/\text{mg}$ of recombinant IFN- β . Such a stronger solubilizing and/or stabilizing agent as SDS also acts to reduce the concentration of non-ionic detergents required to solubilize and stabilize recombinant IFN- β at neutral pH.

Certain non-ionic detergents cannot stabilize/solubilize recombinant IFN- β adequately to prevent precipitation of the recombinant IFN- β without the addition of either glycerol or small amounts of SDS. Exemplary is the Pluronic polyol, Pluronic® F68, which requires such an additional solubilizer and/or stabilizer as SDS or glycerol, to maintain the recombinant IFN- β in solution at the concentration ranges outlined herein. The screening methods outlined below anticipate a protocol that encompasses testing candidate non-ionic detergents with such additional solubilizing and/or stabilizing agents as SDS and glycerol.

The liquid formulations can further comprise an additional stabilizing agent, preferably one or more carbohydrates, and more preferably one or more sugars. Preferred stabilizing agents include, for example, sucrose, dextrose, dextran, mannitol, sorbitol, inositol, fructose, galactitol, xylitol, lactose, and trehalose. More preferred are dextrose and mannitol, and most preferred is dextrose. Additional non-carbohydrate stabilizing agents can include, for example, human serum albumin (HSA) which can be used alone or in combination with a carbohydrate stabilizing agent, and glycine, which is preferably used in combination with a carbohydrate stabilizing agent. Such stabilizing agents are preferably in a concentration (weight/weight) range of from 0.025% to 10%, preferably from 0.05% to 7%, and more preferably from 0.1% to 5%.

The buffer selected to maintain the liquid formulations at a physiologically acceptable pH range is preferably at a concentration from 1 to 50 mM, more preferably from 10 to 25 mM.

The lyophilized formulations of this invention comprise: 1) recombinantly produced, purified IFN- β ; 2) one or more non-ionic detergents or a combination of one or more non-ionic detergents and an additional lyophilizable solubilizing and/or stabilizing agent; 3) a carrier; and 4) a small amount of buffer that provides a physiologically acceptable pH range upon reconstitution.

The lyophilizable additional solubilizing/stabilizing agent is preferably SDS (at the concentrations indicated above for liquid formulations). Glycerol at the concentration ranges employed in the compositions of this invention is considered a non-lyophilizable additional solubilizing and/or stabilizing agent, and, therefore, the concentrations of non-ionic detergents in the lyophilized formulations of this invention are those essentially as outlined above for liquid formulations in the absence of glycerol.

The carrier in said lyophilized formulations can include the carbohydrate stabilizers either alone or in combination as outlined above for liquid formulations and in similar concentration ranges as well as human serum albumin alone or in combination with said carbohydrate stabilizers; however, such a selected carrier

must not only have a stabilizing effect but also must provide bulk to the lyophilized product and is, therefore, herein termed a bulking/stabilizing agent. Preferred bulking/stabilizing agents, at preferred concentration ranges by volume noted-in parentheses, include dextrose alone (1% to 10%, more preferably, 2% to 5%); the combination of dextrose (0.1% to 5%, more preferably 0.1% to 0.2%) with mannitol (0.5% to 5%) more preferably 1% to 3%; or with human serum albumin (HSA) at a concentration from 1% to 5%, more preferably 1% to 2%; and HSA one (preferably from 1% to 5%, more preferably 2.5%). More preferred bulking/stabilizing agents are dextrose, preferably at a concentration of 5%, and a combination of dextrose and mannitol at a concentration ratio by volume of from 1/5 to 1/20 (dextrose/mannitol), and more preferably at a concentration ratio of 1/10. Such combinations of dextrose and mannitol are the most preferred bulking/stabilizing agents of the lyophilized formulations of this invention.

For lyophilized formulations, the buffer is preferably selected from buffers, such as, citrate, maleate, acetate and phosphate, more preferably acetate or phosphate, and still more preferably phosphate, at the same preferred concentration range as indicated above for liquid formulations. Further, for lyophilized formulations, it is preferred that the pH of the composition to be lyophilized (preferably, the recombinant IFN- β desalted pool) be maintained in the preferred range of 3.5 to 6.5, more preferably from 4 to 6.0, and still more preferably 6, to prevent the creation of aggregates during the formulation process because of any time delays necessitated by manufacturing requirements. However, if lyophilization is to be performed within an hour, preferably less than an hour after neutralization (or if the formulation is liquid), the pH of the formulations can range from 3.5 to 9.5, preferably from 4 to 8, more preferably from 5.5 to about 7.5, and still more preferably from 6.0 to 7.2.

Both the liquid and lyophilized formulations of this invention can further contain a minor amount of preservative to enhance chemical stability.

The particular biocompatible polymeric non-ionic detergents employed and the concentration thereof depend mainly on the particular recombinant IFN- β protein or analog to be formulated, the presence or absence of an additional solubilizing and/or stabilizing agent (most notably glycerol), the concentration of the recombinant IFN- β , and pH of the formulation. The optimal concentration of the non-ionic detergent depends upon the pH of the formulation. For example, when Trycol® LAL-12 or Plurafac® C-17 are employed alone as a solubilizer/stabilizer of normal dosage compositions of recombinant IFN- β at pH 5.0, both non-ionic detergents at concentrations as low as 0.01% can solubilize recombinant IFN- β . At pH 7.0, 0.15% Trycol® LAL-12 can solubilize normal dosage IFN- β compositions with only minor aggregation. Plurafac® C-17 at 0.05% concentration can solubilize normal dosage IFN- β compositions at pH 6. In general, the lower the pH of the formulation, the lower the concentration of the non-ionic detergent that is necessary to solubilize/stabilize it.

Further, the concentration of the biocompatible, non-ionic, polymeric detergent varies with the concentration of recombinant IFN- β in the formulation. For example, a high dosage formulation of recombinant IFN- β is that which contains 1 to 2 mg/ml of recombinant IFN- β in the final container vial (2 to 4 x 10⁸ units per mg). A normal dosage formulation has 0.25 mg/ml of recombinant IFN- β in the final container vial (0.5 x 10⁸ units per mg). Whereas, a low dosage formulation has 0.125 mg/ml of recombinant IFN- β in the final vial (0.25 x 10⁸ units per mg). The preferred concentration range of the solubilizer/stabilizer containing one or more non-ionic detergents for a high dosage formulation is from 0.1% to 5%, preferably 0.25% to 3%, and more preferably from 0.5% to 2.0%. For the normal dosage formulations of recombinant IFN- β , the preferred concentration range of the non-ionic detergent is from 0.005% to 1.0%, more preferably 0.075% to 0.5%, and still more preferably from 0.01% to 0.3%.

Lower dosage formulations of recombinant IFN- β would similarly require lower concentration ranges of the biocompatible non-ionic polymeric detergent employed.

A most preferred example of a lyophilized normal dose formulation of this invention comprises:

0.25 mg/ml recombinant IFN- β
0.15% (volume/volume) polyoxyethylene 12 lauryl ether;
20 mM sodium phosphate buffer;
0.2% dextrose; and
2% mannitol.

It is preferred that the polyoxyethylene 12 lauryl ether is Macol® LA-12.

Many of the methods used for the recovery of lipophilic recombinant proteins, such as bacterially produced IFN- β , utilize SDS or similar surfactants as the primary solubilizing agents for the solubilization and isolation of the protein from the cellular material and subsequent acid precipitation to obtain the protein. By further purification techniques carried out at or near neutral pH, the SDS levels in the final protein preparations are reduced to 0.1%. Further removal by diafiltration or desalting techniques in the 3-8 pH range does not follow first order kinetics due to protein SDS interactions which significantly affect the

kinetics of SDS removal. It was found that SDS removal from recombinant proteins promotes protein-protein interactions at the pH range of 3-8 that result in aggregation or precipitation of the protein and consequent loss of activity.

One solution to the problem of such aggregation and precipitation during SDS removal is described in U.S. Patent No. 4,462,940 discussed above, and said patent is herein incorporated by reference. That procedure uses a high pH range (10.5 to 12.5) during diafiltration and desalting. Although the procedure of the '940 patent, as well as any other procedures known in the art, can be used to provide purified recombinant IFN- β for formulation with the stabilizer/solubilizers of this invention, as well as any other recovery, isolation and purification procedures known in the art, the procedures outlined herein are considered the most preferred procedures known to the applicants.

The instant invention solves the problem of recombinant IFN- β aggregation, precipitation and loss of protein activity with the removal of SDS or similar solubilizing agents by methods that further avoid the high alkaline pH range (10.5 to 12.5) of the '940 patent.

Therefore, one aspect of the invention is to avoid such high pH diafiltration or desalting conditions by employing a milder detergent or chaotrope as a transfer component during diafiltration or desalting, preferably desalting, to replace stronger solubilizing agents such as SDS used in the extraction and purification of the recombinant IFN- β from the host microorganisms. Such milder detergent/chaotropes used as transfer components allow for diafiltration or desalting of the recombinant IFN- β to occur at a lower pH range, for example, from pH 8.5 to 10, preferably from 9-9.5. Examples of such detergent/chaotropes for use as transfer components include fatty acid salts having carbon chains of from 10 to 13 carbons, preferably 11 to 12 carbons and most preferably 12 carbons. It is preferred that the fatty acid salt be a laurate salt and most preferred that such laurate salt be sodium laurate.

The concentration range of said transfer component in an elution buffer, preferably a low ionic strength elution buffer, is from 0.05% to 2%, preferably 0.1% to 1% (volume/volume).

In addition to such fatty acid salts, a number of other mild detergent/chaotropes can be used as, for example, urea (5-7 molar, preferably 6M) or, more preferably, guanidine hydrochloride (5-7 molar, preferably 6M).

Further, it is possible to use the non-ionic biocompatible polymeric detergents of this invention as transfer components as well as a solubilizer/stabilizer, providing that the pH and other protocol conditions are optimized for each of the individual non-ionic detergent-containing solubilizer/stabilizers employed.

The pH of the thus purified recombinant IFN- β pool, that is after diafiltration or desalting, preferably desalting, wherein a low ionic strength elution buffer containing a transfer component is employed, is then adjusted to a level of 2 to 4, preferably 3 to 4, and most preferably 3.0, at which point the transfer component, preferably sodium laurate, will precipitate from the solution. The precipitated transfer component is then removed by filtration and/or centrifugation or by other means known to those skilled in the art.

The representative process described in Example 1 *infra* was found to remove the sodium laurate transfer component to levels below the detection limit (that is, approximately 10 moles of laurate per mole of recombinant IFN- β) of an assay employing a standard protein RP-HPLC system with low wavelength UV detection.

The stabilizer/solubilizer containing one or more biocompatible non-ionic polymeric detergents, and optionally another solubilizing and/or stabilizing agent, can then be added to the protein pool. Optionally, said mixture can be held before raising the pH from between 3.5 to 9.5, preferably 4.0 to 8.0, and more preferably 5.5 to 7.5. The amount of time that the solution may be held depends mainly on the particular recombinant IFN- β analog, the particular composition of the stabilizer/solubilizer employed, the exact pH, and the concentrations of recombinant IFN- β protein and stabilizer/solubilizer composition, and typically ranges from 0-240 minutes, preferably 10-180 minutes.

A further aspect of this invention is to provide for extraction, purification and formulation processes wherein the formulated recombinant IFN- β is totally or substantially free of SDS or other less desired solubilizing agents. Said improved processes comprise the use of a non-toxic, milder detergent/chaotrope as the primary solubilizing agent instead of SDS or other strong chemical solubilizing agents during extraction, purification and recovery of the recombinant IFN- β . Such non-toxic detergent/chaotropes include the fatty acid salts discussed above as transfer components, preferably laurate salts, and most preferably sodium laurate (0.5% to 3%, preferably 1-2%). The preferred pH range for such fatty acid salt solubilizers would be between 9 and 10 inclusively, more preferably between 9 and 9.5, inclusively.

It is preferred that when a fatty acid salt, such as sodium laurate, is employed as the primary solubilizing agent, it be in a buffer, such as phosphate, Tris \cdot HCl, or an acetate buffer wherein said buffer is in a molarity range of from 5 to 40 mM, preferably 10-30 mM, and most preferably 20 mM. It is further preferred that the solubilization process be performed slowly over an extended period of time from 1 to 24

hours, and that sonication be performed to promote solubilization. Preferably, reducing agents, such as dithiothreitol (DTT) or β -mercaptoethanol (β -mer), can also be employed with the fatty acid salt to promote solubilization.

Other milder detergent/chaotropes useful as primary solubilizing agent alternatives include guanidine hydrochloride (4-8 M, preferably 5-7M, most preferably 6M) with a reducing agent such as DTT or β -mercaptoethanol (β -mer) (30-70 mM, preferably 40-60 mM, most preferably 50 mM).

When such a fatty acid salt or guanidine hydrochloride is employed as the primary solubilizing agent as an alternative to a detergent, such as SDS, it is preferred that the refractile bodies containing the recombinant interferon- β be at a concentration of about 15 to 5 mg/ml, more preferably 10 to 5 mg/ml, and still more preferably 8 mg/ml. When such primary solubilizing agents are used, the formulations produced are totally or substantially free of SDS.

The use of a fatty acid salt, such as sodium laurate, provides a further method of avoiding highly alkaline conditions and the problems of aggregation, precipitation and loss of activity of the recombinant IFN- β during diafiltration or desalting. When such a fatty acid salt as sodium laurate is used as the primary solubilizing agent during recovery and purification, it is unnecessary to desalt or diafilter the recombinant IFN- β pool as the last step of purification unless there is a need to remove other low molecular weight components such as EDTA. Once the IFN- β has been purified sufficiently, the pH of the recombinant IFN- β pool can be lowered to 2 to 4, preferably 3 to 4, and more preferably 3 to 3.5, at which point the fatty acid salt precipitates. The precipitate can be removed by centrifugation and filtration or by other means known to those skilled in the art. Then the recombinant IFN- β solution can be stabilized with an appropriate solubilizer/stabilizer of this invention, and the pH thereof can be adjusted to that desired for the formulation. If said pH adjustment is to a level above 6.5, preferably in immediate continuous sequence a bulking/stabilizing agent is added, and the solution, optionally pre-filtered and sterile filtered, is lyophilized. As indicated above "immediate" is defined herein to mean less than an hour. If the pH is 6.5 or below, preferably 6 or below, the sequence of adding the bulking/stabilizing agent and lyophilizing the optionally pre-filtered and sterile filtered solution need not be immediate.

It is preferred, when dextrose is used as the carrier for lyophilized formulations of this invention, that the lyophilization cycle be performed as a slow, two-stage ramp. A preferred lyophilization protocol used when dextrose is the carrier is exemplary and follows:

- (a) freeze for 4 hours at -30°C ;
- (b) lyophilize at that temperature for 24 hours;
- (c) ramp at $6^{\circ}\text{C}/\text{hour}$ to $+5^{\circ}\text{C}$;
- (d) hold at $+5^{\circ}\text{C}$ for 12 hours;
- (e) ramp to $+15^{\circ}\text{C}$ at $6^{\circ}\text{C}/\text{hour}$; and
- (f) hold at $+15^{\circ}\text{C}$ for at least 12 hours.

The freezing time of step (a) can be from 2 to 6 hours at a temperature range from -70°C to -20°C . The lyophilizing time of step (b) can be from 12 to 48 hours. The ramping rate for steps (c) and (e) can be from $3^{\circ}\text{C}/\text{hour}$ to $12^{\circ}\text{C}/\text{hour}$ to respective temperature ranges of from -5°C to $+10^{\circ}\text{C}$ (steps c and d) and from $+10^{\circ}\text{C}$ to $+25^{\circ}\text{C}$ (step e). The holding time period for step (d) can be from 6 to 18 hours.

Further, this invention provides for methods of screening known biocompatible non-ionic polymeric detergents, combinations of said detergents, and combinations of one or more of said detergents with another solubilizing and/or stabilizing agent to find stabilizer/solubilizers for the recombinant IFN- β formulations of this invention. A preferred example of such a screening method comprises the steps of:

- (a) passing extracted, purified recombinant IFN- β in 0.1% SDS on a desalting column equilibrated in 0.1% sodium laurate in an elution buffer at pH 9.0-10.0;
- (b) lowering the pH of the eluate with an appropriate acidic agent to pH 2-4;
- (c) adding an appropriate concentration of the candidate biocompatible non-ionic polymeric detergent, or a candidate combination of said detergents, or of a candidate detergent or combination of said detergents with another solubilizing and/or stabilizing agent to the desalted pool;
- (d) adjusting the pH to 3.5 to 9.5 with an appropriate basic agent; and
- (e) allowing said solution to stand for 24 hours at pH 3.5 to 9.5.

The term purified recombinant IFN- β in the screening step (a) above preferably refers to recombinant IFN- β that has been purified according to the procedures described in Example 1 below and outlined in Figures 2A and 2B through the sizing step of the G-75 column. However, such a screening method is exemplary and other screening methods employing other methods of purifying recombinantly produced IFN- β are within the scope of this invention.

If the candidate stabilizer/solubilizer maintains the recombinant IFN- β in solution at pH 3.5 to 9.5 over a 24-hour period, it is considered for inclusion in the prototype formulations of this invention.

A preferred screening process includes the step of filtering from the desalted recombinant IFN- β pool the precipitated sodium laurate which precipitates in step (b).

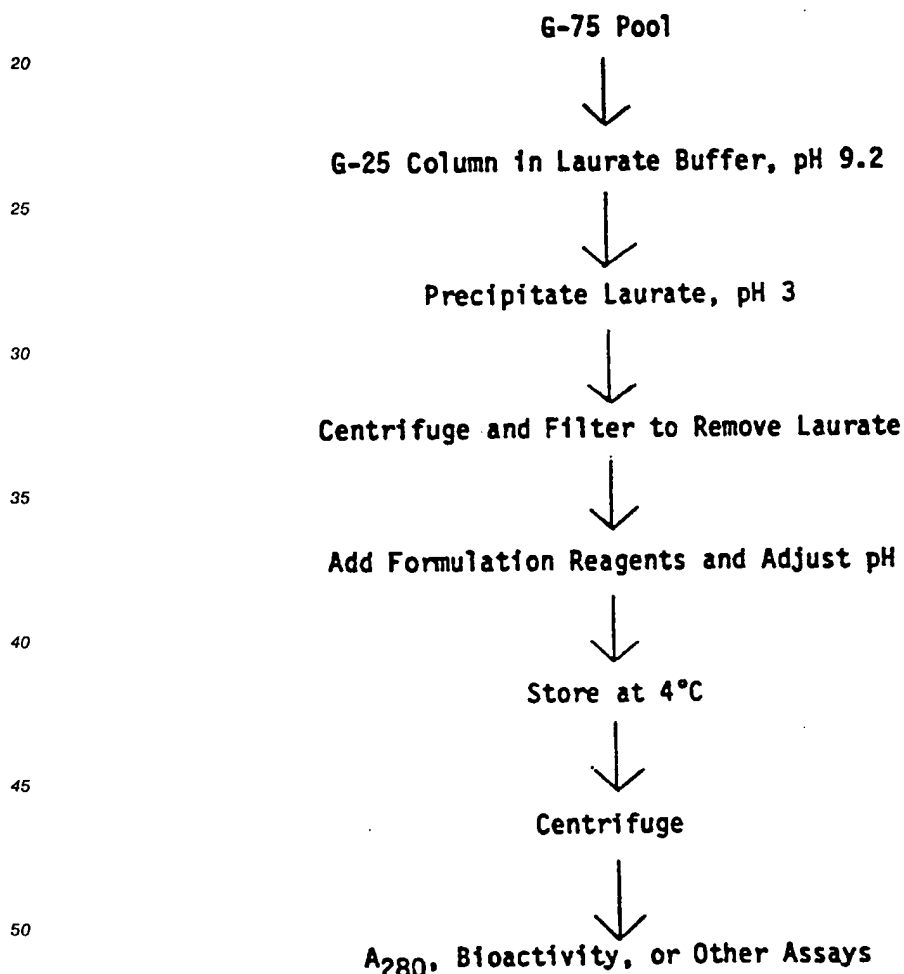
A further preferred screening method of this invention is that wherein the pH range for step (a) is 9-9.5, for step (b) is 3-4 and other steps (d) and (e) are performed at the pH range of about 4 to about 8. A still further preferred screening method is that wherein the pH range for step (a) is 9-9.2, for step (b) is 3 to 3.5, and wherein steps (d) and (e) are performed at the pH range of 5.5 to 7.5.

The elution buffer of step (a) is preferably Tris-HCl at pH 9 to 9.8; borate at pH 9.0 to 9.8; sodium phosphate at pH 9.0 to 9.8; acetate at pH 9.0 to 9.8; or sodium pyrophosphate at pH 9.0 to 9.8.

The following Screening Flow Chart graphically illustrates further exemplary methods of screening formulation reagents for the recombinant IFN- β pharmaceutical compositions of this invention. The terms G-75 and G-25 are gel filtration columns, which are positioned in a representative recombinant IFN- β purification process according to Example 1, *infra*.

Screening Flow Chart

Process for Preparing Recombinant IFN- β for Formulation Studies



To analyze prototype formulations, ultracentrifugation is used as a simple method of detecting the presence of high molecular weight aggregates. Such candidate formulations can also be screened by SDS-PAGE under non-reducing conditions and Western blots.

The preferred range of recombinant IFN- β in such formulations is from 0.05 mg/ml to 10 mg/ml, more preferably 0.1 mg/ml to 5 mg/ml, and still more preferably 0.1 mg/ml to 2.0 mg/ml.

A preferred process for preparing lyophilized IFN- β formulations of this invention comprises the steps of: (a) extracting the recombinant beta-interferon from the disruptate of a host organism transformed to produce the protein; (b) purifying the recombinant IFN- β protein using as the last purification step a desalting step at a pH range of 8.5 to 10 employing 0.05 to 2% sodium laurate as a transfer component; (c) lowering the pH of the desalted pool with an appropriate acidic agent to a pH of 2 to 4; (d) removing the precipitated sodium laurate by centrifugation and filtration; (e) adding to the desalted pool an effective amount of one or more non-ionic biocompatible polymeric detergents, or a combination of one or more biocompatible non-ionic polymeric detergents with another solubilizing and/or stabilizing agent to stabilize/solubilize the recombinant IFN- β ; (f) adjusting the pH of the pool with an appropriate basic agent to a range of between 3.5 and 9.5; (g) adding an effective amount of a bulking/stabilizing agent; (h) pre-filtering and sterile filtering the solution; and (i) lyophilizing the formulation.

Preferably, if the pH adjustment in step (f) is to a level above 6.5, steps (f) through (i) are carried out in rapid, continuous succession to avoid the creation of recombinant IFN- β aggregates. It was found that freezing the sample immediately after neutralization to pH 6.5 and above in dry ice and ethanol did not produce aggregates, whereas freezing the samples slowly by placing the room temperature samples at -20 °C caused some degree of aggregation. When formulations were neutralized to a pH at or above 6.5 four hours before lyophilization, a 30% to 40% increase in the amount of aggregation was obtained when compared to samples that were neutralized immediately (within an hour) before lyophilization. Therefore, it is preferred that lyophilization be performed immediately after neutralization to pH 6.5 and above which results in very low levels of aggregates. However, when the neutralization step (f) is to pH 6.5 or below, more preferably at or below pH 6.0, there is no significant aggregation in samples left at room temperature for 24 hours before lyophilization.

Further, preferably, the desalting step is carried out at a pH range of from 9 to 9.5. Also preferably, the pH range of step (c) is 3 to 4, and more preferably pH 3.0 and for step (f) is 4-8, more preferably 4.0 to 6.5, and still more preferably 4.0 to 6.0. Further, preferably, the pH range for step (c) is 3.5, and for step (f) is from 5.5 to 6.5.

In preferred embodiments of the invention, the non-ionic biocompatible polymeric detergent stabilizer/solubilizers or combinations thereof are Laureth 12 detergents, Plurafac® C-17, Laureth 12/Nopalcol® in a 2:1 volume ratio, or Triton® X305 or X405/Nopalcol® in a 2:1 volume ratio. Further, preferred is the concentration of 0.01% of any of the preferred non-ionic detergents (including Pluronic® F68) with glycerol at a concentration of about 25%.

For purposes of practicing the present invention, bacteria are the preferred microorganism hosts, with *E. coli* being the most preferred.

In general, the recovery, purification and formulation processes herein involve fermenting the host organism transformed to express the recombinant IFN- β , disrupting the cell wall and cell membrane of the host organism, separating the refractile material containing the recombinant IFN- β from the rest of the cellular debris, solubilizing the refractile material in an aqueous buffer under reducing conditions, extracting the recombinant IFN- β with 2-butanol or 2-methyl-2-butanol, subjecting the extracted recombinant IFN- β to chromatographic purification, and then diafiltering or desalting, preferably desalting, the recombinant IFN- β to remove the solubilizing agent optionally using a suitable transfer component and formulating as described above.

Figures 1A and 1B outline a preferred embodiment of the invention wherein sodium laurate is used as the primary solubilizing agent and as a transfer component.

Figures 2A and 2B outline preferred embodiments of this invention wherein sodium dodecyl sulfate (SDS) is used as the primary solubilizing agent, and sodium laurate is used as a transfer component during a desalting step on a Sephadex® G-25 desalting column. Further, Figures 2A and 2B illustrate the details of the individual process steps of the preferred embodiment of the present invention including the culture of the transformed microorganisms in an appropriate fermentation medium through the final step where the purified recombinant IFN- β is stabilized and then lyophilized and reconstituted into therapeutic formulations. The individual process steps of such an example of one embodiment of the instant invention are summarized as follows:

- (a) growing the transformed bacterial hosts in an appropriate fermentation medium;
- (b) concentrating the bacteria in the fermentation medium by cross-flow filtration, centrifugation or other conventional methods;
- (c) disrupting the cell wall and cell membrane of the bacteria;

(d) removing greater than 99% by weight of the salts from said disruptate by diafiltration or centrifugation;

(e) redispersing the desalted disruptate;

(f) adding a material to the disruptate to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate;

(g) separating the refractile material from the cellular debris by high-speed centrifugation;

(h) solubilizing the refractile material in an aqueous buffer containing a reducing agent;

(i) organically extracting the solubilized refractile material, preferably with 2-butanol or 2-methyl-2-butanol;

(j) isolating said refractile material from the extractant, preferably by employing an acid precipitation step followed by centrifugation;

(k) solubilizing the resulting recombinant IFN- β particle pellet with distilled water or with an aqueous solution of SDS at a IFN- β to SDS ratio of about 1:3;

(l) adjusting the pH of the solution to about 9.5 and reducing the solubilized recombinant IFN- β ;

(m) purifying the reduced recombinant IFN- β by chromatography;

(n) oxidizing the recombinant IFN- β from step m;

(o) further purifying the oxidized recombinant IFN- β by gel chromatography and collecting the eluate containing the purified recombinant IFN- β ;

(p) desalting the purified recombinant IFN- β eluate in a desalting column equilibrated and run in 0.1% sodium laurate in 10mM Tris-HCl at pH 9.2;

(q) lowering the pH of the eluate quickly to pH 3.0 with an appropriate acidic agent;

(r) centrifuging and filtering the recombinant IFN- β pool;

(s) adding an effective amount of a non-ionic biocompatible polymeric detergent-containing solubilizer/stabilizer;

(t) adjusting the pH of the solution to near physiological pH;

(u) adding an appropriate bulking/stabilizing agent in a concentration of from 0.25% to 10%;

(v) filtering the solution;

(w) immediately lyophilizing the recombinant IFN- β sample; and

(x) reconstituting the lyophilized recombinant IFN- β sample, if desired.

Ten mM dithiothreitol may be optionally included in the initial solubilization step, and the mixture may be heated to about 50 °C for about 10 minutes. In addition, the recombinant IFN- β is preferably oxidized so that its cysteine residues are bridged to form cystines, as described by U.S. Patent No. 4,530,787 to Shaked et al., using o-iodosobenzoic acid solution or by U.S. Patent No. 4,572,798 to Kothe et al., entitled "Method for Promoting Disulfide Bond Formation in Recombinant Proteins," using copper chloride. Preferably, o-iodosobenzoic acid is employed for the oxidation.

In another preferred embodiment of the process of the invention, the disrupted cells are treated to isolate and purify the recombinant interferon- β protein, and then the following steps are carried out: (a) desalting the recombinant IFN- β protein by G25 chromatography at pH 9-9.2 employing an elution buffer containing 0.05% to 2% sodium laurate as a transfer component; (b) adjusting the pH of the desalted pool to 3.5; (c) centrifuging and filtering to remove the precipitated transfer component; (d) adding the stabilizer/solubilizer composition to the desalted pool; (e) adjusting the pH of the recombinant IFN- β mixture to neutral pH; (f) adding an effective amount of a bulking/stabilizing agent; (g) filtering the pool; (h) immediately lyophilizing the recombinant IFN- β protein sample upon neutralization and bulking/stabilizing addition; and (i) reconstituting the lyophilized recombinant IFN- β protein sample, if desired.

EP 206,828 details procedures for extracting and purifying recombinant protein, such as IFN- β , which are deposited within the microbial host in refractile bodies. Said disclosure focuses on the isolation of the refractile materials by front-end processes which are termed either "abbreviated" or "expanded." A synopsis of said procedures follow.

The transformed microorganisms are grown in a suitable growth medium, typically to an optical density (OD) of at least 30 at 680 nm, and preferably between 20 and 40 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The medium is an aqueous medium containing compounds that fulfill the nutritional requirements of the microorganism. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (See Review of Medical Biology, Lange Medical Publications, 14th Ed pp. 80-85 (1980).) In expression vectors involving the trp promoter, the tryptophan concentration in the medium is carefully controlled to become limiting at the time IFN- β expression is desired. Growth media for E. coli are well known in the art.

After the cells are harvested from the culture, they may be concentrated, if necessary, to 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by cross-flow filtration, centrifugation, or other conventional methods. Preferably a compound which is non-toxic to humans, such as 1-octanol, in an amount of about 1% by weight of total components, is added to the fermenter before or during cell concentration to ensure that no viable recombinant organisms remain before containment is broken.

Following concentration of the harvested culture, the cell membranes of the microorganisms are disrupted. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. Preferred methods are sonication or homogenization with a homogenizer. The end point of the disruption step can be determined by monitoring the optical density with the absorbance at 260 nm of the suspension typically increasing with cell lysis. In any event, the disruption should break substantially all of the cells so that substantially no intact cells are carried through to the solubilization step. Before the disruption, the pH of the liquid phase of the concentrate is adjusted, if necessary, to a level that facilitates removal of *E. coli* proteins in subsequent steps, while retaining the heterologous protein as an insoluble complex in the cellular debris.

After the cells have been disrupted, deionized water is preferably added to the disruptate and greater than 99% by weight of the salts are removed therefrom. The removal of these salts to reduce the ionic strength of the disruptate may be accomplished by diafiltration using deionized water to flush out the ions or by centrifuging to pellet the cellular debris and refractile bodies followed by resuspension in deionized water.

After the salts are essentially removed, optionally a compound such as 1-octanol may be added to the desalted disruptate, if not added earlier, to ensure that no viable recombinant organisms remain. The desalted disruptate is again disrupted as described above for the initial disruption.

After redispersion, density or viscosity is increased and/or a gradient is created during centrifugation in the liquid within the disruptate by adding a material to the disruptate.

In the final step of the abbreviated "front-end" process to recover the refractile bodies, the refractile bodies containing the desired protein are separated from the cellular debris by high-speed centrifugation. By "high-speed centrifugation" is meant spinning the suspension in a centrifuge at 10,000 to 40,000 times gravity, preferably 10,000-20,000 x g, for a suitable time period depending on the volume, generally about 10 minutes to seventy-two hours. The pellet resulting from the centrifugation is called the "particle pellet" or "particle paste." The abbreviated front-end process is most preferably used when sodium laurate is the primary solubilizing agent.

In an alternative, expanded "front-end" process to recover the refractile bodies, the particle pellet obtained from the last centrifugation step of the abbreviated front-end process, is solubilized, reduced and then extracted from the aqueous medium with 2-butanol or 2-methyl-2-butanol. The extractant phase is then precipitated with an acid and centrifuged to produce a "final pellet" or "final paste" which is then further purified as indicated.

The alternative, expanded front-end process is distinguished from the abbreviated front-end process in that it comprises several additional steps as follows: solubilizing the refractile bodies under reducing conditions; organically extracting the solubilized refractile material; and isolating said refractile material from the extractant. Essentially, the enhanced purity of the final pellet as opposed to the particle pellet lessens the purifying burden of downstream processing. There is an interdependence between the choice of the front-end process and later process purification steps to achieve the desired purity level for the final product. Once the choice of the particular front-end recovery of the refractile bodies has been made, one skilled in the art can pick and choose the alternative purifying steps outlined below to achieve the desired purity level of the final product.

Whether the abbreviated or expanded front-end process is utilized to recover the refractile bodies containing the recombinant IFN- β , the next step in purification is solubilizing either the particle or final pellet containing the refractile material. The following solubilizing agents can be used: sodium dodecyl sulfate (SDS), sodium laurate, urea, sodium dodecyl sulfonate, sodium decyl sulfate, sodium tetradecyl sulfate, sodium tridecyl sulfonate, sodium dodecyl N-sarcosinate, sodium tetradecyl N-sarcosinate, sodium dioctyl-sulfosuccinate, and guanidine hydrochloride. Preferred solubilizing agents are SDS, sodium laurate or guanidine hydrochloride. Most preferred is sodium laurate or guanidine hydrochloride at concentrations and under the conditions indicated above to eliminate the possibility of SDS in the final container product.

The solubilizing agent is in an aqueous buffer, preferably phosphate buffered saline. The preferred percentage of the solubilizing agent is in the range of 1% to 5% (w/v). (Percentages herein reflect weight to volume ratios.) The preferred solubilizing solutions are phosphate buffered saline with 1-2% sodium laurate (20mM NaPO₄) at pH 9-10 and 6 M guanidine hydrochloride in 50 mM β -mercaptoethanol. Sonication is

preferably employed when either sodium laurate or guanidine • HCl is employed as the solubilizing agent to promote solubilization.

Reducing agents that can be employed during the solubilization step include: β -mercaptoethanol (β -mer), glutathione, cysteine and dithiothreitol (DTT). DTT and β -mer are the most preferred reducing agents. It is preferred that reducing agents be employed when either sodium laurate or guanidine hydrochloride is used as the primary solubilizing agent.

The solubilization will typically be carried out at temperatures in the range of 20 °C to 25 °C with mixing to facilitate contact between the solid phase and the solubilizing medium. Optionally, a reduction step may be carried out at this point. The pH, if necessary, may be adjusted to a range of 8.5 to 10, most preferably approximately 9. The suspension may be heated to 50 ± 5 °C for 5 to 15 minutes under nitrogen. The reaction mixture would then be cooled to approximately 25 °C.

The solubilization is considered complete when the sample has sat 15 minutes or the solution turns translucent. Optionally at this point, the insoluble material may be separated by centrifugation or filtration after completing the solubilization.

After the protein is solubilized, the resulting suspension may optionally be centrifuged at 10,000-40,000 x g, preferably 10,000 to 15,000 x g, to obtain a pellet containing, inter alia, additional host (e.g., *E. coli*) proteins, notably including certain contaminants that have molecular weights very close to that of the desired protein. The exact speed of centrifugation is not critical, as most of the insoluble material will come out, even at low speeds. The pellet is discarded and the supernatant containing the desired protein is retained and processed to recover the desired protein.

If a reduction step was not carried out during the solubilization, the next step in the process would be a reduction of the solubilized refractile body protein. A preferred reducing agent is dithiothreitol (DTT). Reduction conditions may also include the addition of a chelating agent such as ethylenediaminetetraacetic acid (EDTA).

The next step in the process is to separate the protein in the supernatant from any host contaminants remaining after the centrifugation or filtration and optimally from the solubilizing agent. Gel filtration chromatography, reverse-phase high performance liquid chromatography (RP-HPLC), or a combination of gel filtration chromatography and RP-HPLC, can be used. The gel filtration chromatography is preferably carried out in two stages that remove both pyrogenic components and protein contaminants having molecular weights higher or lower than that of the protein. Gels that are capable of fractionating the solution to permit separation of the protein from these contaminants are commercially available. Sephacryl® S-200 is a preferred gel for removing the higher molecular weight components and Sephadex® G-75 or G-100 gels are preferred for removing the low molecular weight contaminants. The gel filtrations will typically be run in buffered solutions (pH 5.5 to 7.0) containing 0.1% to 1.5% solubilizing agent and 0.5 to 10 mM reducing agent. The column will be sized to permit suitable resolution of the desired components.

RP-HPLC is an alternative to gel filtration. Also, RP-HPLC is capable of removing molecules from the solution that have molecular weights close to the protein and cannot, therefore, be removed completely by gel filtration. In addition, contaminants such as bacterial endotoxin are also removed effectively by RP-HPLC. Therefore, RP-HPLC may also be used as a final purification step after gel filtration.

An alternative and preferred procedure is to oxidize selectively, under controlled conditions, the recombinant IFN- β protein after it has been separated by gel filtration, as described in U.S. Patent No. 4,572,798 to K. Kothe et al. (using an oxidation promoter containing a Cu^{+2} cation) and in U.S. Patent No. 4,530,787 to Z. Shaked et al. (using *o*-iodosobenzoic acid). The oxidized product is purified by RP-HPLC or gel filtration followed by RP-HPLC.

It is preferred in carrying out the process of this invention that the last step of purification before stabilization of the formulation is a desalting step employing a transfer component, such as sodium laurate at a pH range of 8.5 to 10. The purity of the protein after the chromatography step(s) is at least 95% and higher, and usually at least 98%. This highly pure material contains less than 2 ng endotoxin, usually less than 0.01 ng endotoxin, per 100,000 units protein bioactivity.

The formulation of the protein in accordance with this invention is then carried out as described in detail above. It may be carried out as a separate operation using purified, selectively oxidized protein or in an operation that is integrated with the purification of the selectively oxidized protein. In the latter case, the starting material for the formulation is a protein-containing product from a RP-HPLC treatment of the selectively oxidized product. Preferably a product selectively oxidized by the RP-HPLC product (pool) will comprise a solution of the protein in a water-organic solvent mixture. The nature of the organic solvent will depend upon the solvent system used in RP-HPLC. Examples of systems that may be used are combinations of an organic acid such as acetic acid, trifluoroacetic acid or heptafluorobutyric acid, and an organic solvent such as propanol or acetonitrile.

Further, other conventional solid non-protein bulking/stabilizing agents that are used in pharmaceutical tablet formulation may be used as the carrier. These materials are water soluble, do not react with the recombinant IFN- β protein, and are themselves stable. They are also preferably non-sensitive to water, that is, non-hygroscopic. Specific examples of such other candidate carriers include starches and starch hydrolysates derived from wheat, corn, rice, and potatoes, as well as micro-crystalline celluloses.

The unit dosage amounts, that is, 0.125 to 2 mg, preferably 0.25 to 1 mg, of the recombinant beta-interferon in the solution are dispensed into containers, the containers are capped with a slotted stopper, and the contents are lyophilized using conventional freeze-drying conditions and apparatus.

The lyophilized formulations may be reconstituted by injecting into the vial a conventional parenteral aqueous injection such as distilled water for injection, Ringer's solution injection, D5W glucose solution, Hank's solution injection, dextrose injection, dextrose and salt injection, physiological saline injection, or the like, preferably a diluent, more preferably a NaCl solution which yields an isotonic solution upon reconstitution. The injection should be added against the side of the vial to avoid excess foaming. The amount of injection added to the vial will typically be in the range of 1 to 5 ml, preferably 1 to 2 ml.

The reconstituted formulation prepared as described above is suitable for parenteral administration to humans or other mammals in therapeutically effective amounts (i.e., amounts which eliminate or reduce the patient's pathological condition) to provide therapy thereto. IFN- β therapy is appropriate for anti-cancer, anti-viral and anti-psoriasis treatment.

The formulations of this invention are useful for parenteral administration, for example, intravenous, intrathecal, subcutaneous, intraperitoneal, intramuscular, intraorbital, ophthalmic, intracapsular, intraspinal, intrasternal, intranasal aerosol, scarification, and also, for oral administration. The preferred routes of administration are by intramuscular, subcutaneous and intravenous injection. The use of non-ionic detergents are especially preferred for topically administered formulations because of their ability to penetrate the skin surface.

Example 1

A recombinant analog IFN- β designated IFN- β_{ser17} was recovered from *E. coli*. The amino acid sequence of this recombinant IFN- β is different from that of native human IFN- β in that the cysteine at position 17 has been changed to serine. The strain of IFN- β_{ser17} -producing *E. coli* (K12/MM294-1) carrying plasmid pSY2501 used in this example was deposited at the American Type Culture Collection on November 18, 1983 under accession number 39,517. Said analog is described in U.S. Patent Nos. 4,518,584 and 4,588,585 assigned to Cetus Corporation.

The *E. coli* thus transformed were grown in a 1000-liter fermentor at 37°C. The dissolved oxygen was maintained at about 40% by, as necessary; (1) increasing agitation; (2) adding air; and (3) adding oxygen.

Once the fermenter was filled with water to the operating volume, the following trace elements were added:

| | |
|---|------------|
| ZnSO ₄ • 7H ₂ O | 72 mM |
| MnSO ₄ • 4H ₂ O | 30 μ M |
| CuSO ₄ • 5H ₂ O | 3 μ M |
| Na ₃ citrate • 2H ₂ O | 1.5 mM |
| KH ₂ PO ₄ | 21 mM |
| (NH ₄) ₂ SO ₄ | 72 mM |

The fermenter feed and addition vessels were then sterilized according to standard operating procedures. Then the following sterile additions were made:

| | |
|---------------------------------------|-------------|
| MgSO ₄ • 7H ₂ O | 20 mM |
| FeSO ₄ • 7H ₂ O | 100 μ M |
| L-tryptophan | 70 mg/L |
| thiamine • HC1 | 20 mg/L |
| glucose | 5 g/L |

The fermenter was cooled and inoculated with frozen or seed *E. coli* culture at 2 mg/L. A glucose feed was employed to maintain the glucose concentration between 5-10 g/L. At approximately 15 hours after

fermentation was begun, the pH was adjusted with KOH to 6.8. Optical density measurements and residual glucose measurements on samples were taken at 14-16 hours and approximately one hour intervals thereafter.

Induction of IFN- β_{ser17} production by depletion of L-tryptophan from the culture medium occurred at about $\text{OD}_{680} = 10$ followed by the addition of casamino acids to a final concentration of 2% at $\text{OD}_{680} = 15$. The cultures were harvested when glucose consumption reached 40 ± 6 g/l.

The refractile bodies containing the IFN- β_{ser17} protein were then isolated. The harvested material was concentrated about 5-10 fold by circulating the harvest material under pressure through UF cross-flow filtration cartridges with a 100K molecular weight cutoff. Cells were disrupted by 3 passes through a Manton-Gaulin high-pressure homogenizer at 6,000 to 8,000 psig.

EDTA was added to the disruptate to a final concentration of 5 mM. The suspension was then diafiltered against 5 volumes of deionized water.

EDTA was then added to a final concentration of 2 mM. Octanol was added to 1% (v/v) to kill any residual live bacteria in the diafiltered product. The suspension was redispersed by passing it twice through the Manton-Gaulin high-pressure homogenizer at 6,000-8,000 psig.

Sucrose was added to the redispersed to a final concentration of 23% (wt/wt), creating a final density gradient between 1.1 and 1.25 g/ml. The mixture was centrifuged at 10,000 to 15,000 x g, and the particle pellet or paste was collected. A temperature of at least 20°C was maintained prior to and during centrifugation.

The particle pellet was then solubilized in phosphate buffered saline with 2% SDS. Solid DTT and EDTA were added to a final concentration of 10 mM and 2 mM, respectively. The suspension was heated to $50 \pm 5^\circ\text{C}$ for 10 minutes under nitrogen. The reaction mixture was then cooled to approximately 25°C, and then the pH of the mixture was adjusted to 7.4.

A volume of 2-butanol equal to the total volume of the suspension was measured. The suspension and organic solution were pumped separately but simultaneously at flow rates of 1.1 to 1.3 liters per minute through a static mixer and then into a continuous centrifuge (Westfalia at approximately 11,770 xg) for phase separation. The 2-butanol-rich phase containing the IFN- β_{ser17} was collected (Organic Extract).

The 2-butanol extract was mixed with 2.5 volumes of 0.1% SDS in phosphate-buffered saline. Solid DTT was added to a final concentration of 2 mM. The pH of the organic extract/buffer solutions was adjusted to 6.2 ± 0.1 with glacial acetic acid (Acid Precipitate).

The mixture was then centrifuged (Sharples centrifuge at 13,200 x g) for approximately 2-6 hours, the supernatant was decanted, and the final pellet was then collected (Final Pellet) containing approximately 81% IFN- β . The final pellet containing the refractile material was then further purified by downstream processing.

The final pellet was then re-suspended with 2% SDS in 50 mM phosphate buffer and 5 mM EDTA. Solid DTT was added to a final concentration of 20 mM, and the pH was adjusted to 8.5 with NaOH. The suspension was heated to $50 \pm 5^\circ\text{C}$ for 10 minutes under nitrogen, and then cooled to approximately 25°C. The pH was then adjusted to a pH of 5.5 with glacial acetic acid, and the solution was filtered through a 0.65 μm filter.

The filtrate was then processed by pre-column chromatography by loading a Sephacryl® S200 column and collecting fractions into clean, depyrogenated vessels using an elution buffer that is composed of 50 mM acetate, pH 5.5, 1 mM EDTA and 1% SDS. The fractions containing the IFN- β monomer were pooled.

The pre-column pool was then concentrated by using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The concentrated pre-column pool was then oxidized using o-iodosobenzoic acid (IBA). The oxidation was effected by adding equimolar amounts of protein and IBA into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. A 20 μM excess of IBA was present at the end of the oxidation. The pH was controlled at 9.0 ± 0.1 with NaOH during oxidation, and adjusted to 5.5 ± 0.2 with glacial acetic acid when the oxidation was completed.

The IFN- β protein was then concentrated using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The protein was then loaded onto the main column (Sephacryl® S200-A), and fractions were collected into clean, depyrogenated vessels using an elution buffer that is composed of 50 mM acetate, pH 5.5, 1 mM EDTA and 0.1% SDS.

A SDS-PAGE was performed on samples from each fraction tube starting from the beginning of the peak to be pooled to the end of the peak. Using the SDS-PAGE results, the fractions containing no high molecular weight contaminants were determined. Those fractions were then pooled.

The main column pool was then concentrated by using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The above procedure performed with the main column was repeated on a Sephadex® G-75 column. Using the SDS-PAGE results, the fractions containing neither low nor high molecular weight contaminants were pooled.

The desalting step was then performed at pH 9.2 wherein 0.1% sodium laurate was used as a transfer component as follows. The pH was adjusted with an appropriate basic agent such as 1mM NaOH.

A Sephadex® G-25 column was then equilibrated with 0.1% sodium laurate in 10 mM Tris-HCl, pH 9.2 and loaded with the Sephadex® G-75 pool containing 0.1% SDS. Using the process chromatogram, the IFN- β_{ser17} peak was collected. The pH of the eluate was then lowered quickly with 1.0 N HCl to pH 3.0, which precipitated the sodium laurate, but left the IFN- β_{ser17} in solution.

The mixture was centrifuged at 35,000 x g for 30 minutes and the supernatant was filtered through a 0.22 micron nitrocellulose filter. SDS concentration was assayed by acridine orange. [Sokoloff et al., "Rapid Spectrophotometric Assay of Dodecyl Sulfate Using Acridine Orange," *Anal. Biochem.*, 118:138-141 (1981).]

The recovery of the G-25 pool was above 85%, and the SDS concentration was reduced to less than 10 $\mu\text{g}/\text{mg}$.

The filtered supernatant was then stabilized by adding 0.15% Trycol® LAL-12. The pH of the formulated product was then raised to about 7.0 ± 0.3 with NaOH. The bulking/stabilizing agent, 5% dextrose was then added. The solution was then pre-filtered and sterile filtered through 0.45 and 0.22 micron nitrocellulose filters, respectively. Immediately thereafter the correct dosage amounts of the IFN- β_{ser17} 0.25 mg containing 5×10^8 units, were aseptically filled into sterilized vials with sterilized stoppers under sanitary and sterile conditions that were carefully monitored. The vials were then quickly placed in a lyophilizer where appropriate thermocouples were attached. The vials were frozen to between -35° and -45°C . The lyophilization cycle was completed, and the vials were mechanically sealed under a vacuum.

Example 2

This example follows procedures similar to those outlined in Example 1 with the main difference being that sodium laurate is employed as the primary solubilizing agent rather than SDS. The other differences are indicated below.

The procedures of this example correspond essentially to those outlined in Figures 1A and 1B. Therefore, at the point of paste solubilization, instead of 2% SDS, 1-2% sodium laurate in 20 mM disodium phosphate plus a reducing agent (50 mM DTT) is used to solubilize the particle pellet. The pH was adjusted to about 9 with NaOH. Sonication is preferably employed to promote solubilization.

Also, differing from Example 1, is the elimination after the first reduction of the steps of the expanded front-end process, including the Organic extraction, Acid precipitation, Centrifugation, Acid precipitate solubilization, and second Reduction. The buffer is 10 mM Tris • HCl rather than 50 mM acetate for the gel chromatography steps on the Sephacryl® S200 columns and on the Sephadex® G-75 column. Further, such gel chromatography is run at pH 9.2 rather than at pH 5.5, as are the Concentration steps.

The elution buffer on the first S200 column contains 1-2% sodium laurate rather than 1% SDS as in Example 1. The elution buffer of the second S200 column contains 0.1-0.5% sodium laurate rather than 0.1% SDS, and the buffer for the G-75 column contains 0.1%-0.5% sodium laurate rather than 0.1% SDS.

During the oxidation step, 1-2% sodium laurate is used rather than 0.1% SDS.

As in Example 1, the IFN- β_{ser17} is formulated by lowering the pH of the eluate to about 3, centrifuging and filtering to remove the precipitated sodium laurate, adding an effective amount of one or more biocompatible non-ionic polymeric detergents or a combination of such non-ionic detergents and another solubilizing agent, adjusting the pH to about physiological pH, adding an appropriate bulking/solubilizing agent, preferably dextrose or mannitol, pre- and sterile filtering the pool and immediately lyophilizing the formulated, neutralized product.

The primary advantage of the procedures of this example is to arrive at a formulation that is completely or substantially free of SDS.

Example 3

The procedure outlined in Example 1 wherein sodium laurate was used as a transfer component on the G-25 desalting column was repeated using a Pharmacia K50 column (1700 ml. bed volume). Fifty ml of the G-75 eluate (approximately 3.64 mg/ml of IFN- β) was loaded on the K50 column equilibrated in 0.1% sodium laurate in 20 mM sodium phosphate and run at pH 9.2. The recovery of the IFN- β was 68%, and

the SDS level was less than 10 µg/mg of protein or less than 1 ppm. At pH 3, the IFN-β was soluble with no detergent as shown by UV scan.

Example 4

The following IFN-β formulations were prepared according essentially to the procedure of Example 1 where 0.1% sodium laurate was used as a transfer component. No bulking/stabilizing agents, however, were added to the formulations, and they were not lyophilized. Precipitation was checked visually and by UV scan. Table I shows the results.

TABLE I

| | |
|--------------------------|--|
| Durfax® 80/SDS | 0.1%/200 µg/mg Polysorbate Remains soluble for days at neutral pH by UV scan. |
| Trycol® LAL(12)/Nopalcol | 0.1%/0.05% POE(12) lauryl ether/PEG(400) monooleate Remains soluble for days at pH 7.0 by UV scan (20 µg/mg SDS). |
| Triton® X305/Nopalcol | 0.1%/0.05% C ₈ H ₁₇ phenyl(OCH ₂ CH ₂) ₃₀ OH/PEG(400) monooleate Remains soluble overnight at pH 7.0 for days at pH 5.0 by UV scan. The detergent solution without IFN-β is slightly cloudy (45 µg/mg SDS). |
| Trycol® LAL(12) | 0.1% POE(12)lauryl ether Soluble at 6.5 and 6.0. Better UV scan at 6.0 (10 µg/mg SDS). |
| Plurafac® C-17 | 0.1% modified oxyethylated straight chain fatty alcohol. Soluble at pH 6.0 for days (10 µg/mg SDS) |

Example 5

Ultracentrifugation Data

Ultracentrifugation is a simple method of detecting the presence of high molecular weight aggregates and oligomers in the formulations of the invention. Ultracentrifugation was performed in a Beckman L8-70 using a type 70.1 Ti rotor. Five milliliter samples of the formulated product, lyophilized and reconstituted, were spun at 55,000 rpm for one hour. The supernatant was measured by absorbance at 280 nm. Said absorbance was then compared to that prior to centrifugation. Table II below shows the comparative results of a number of formulations, some of which do not contain biocompatible non-ionic polymeric detergents and are inserted for comparative purposes.

TABLE II

| Ultracentrifugation 55,000 rpm -- 60 min. | | | |
|---|-------------------------|------------------------|------------------|
| Samples | A ₂₈₀ Before | A ₂₈₀ After | Percent Recovery |
| 1.25% HSA (human serum albumin) | 1.696 | .543 | 32% |
| 0.1% SDS pH 7.5 | .257 | .238 | 93% |
| 0.1% Laurate pH 7.5 | .408 | .392 | 96% |
| 0.1% Durfax® 80/150 µg/ml SDS pH 7.0 | .306 | .116 | 39% |
| 0.1% Durfax® 80/200 µg/mg SDS pH 7.0 | .419 | .442 | 105% |
| 0.1% Triton® X305/0.05% Nopalcol pH 7.0 | .140 | .061 | 46% |
| 0.1% Trycol®/0.05% Nopalcol pH 7.0 | .357 | .387 | 108% |
| 0.1% Plurafac® C-17 pH 6.0 | .530 | .456 | 86% |
| 0.1% Trycol® pH 6.5 | .475 | .415 | 87% |

The results in Table I indicate that the formulations of IFN-β with 0.1% Trycol® at pH 6.5, 0.1% Plurafac® C-17 at pH 6.0, 0.1% Trycol®/0.05% Nopalcol® at pH 7.0, and 0.1% Durfax® 80/200 µg/mg SDS

at pH 7.0 all had significantly less high molecular weight species than the HSA formulation.

Example 6

The IFN- β formulations listed in Table 1 and IFN- β formulations with 0.1% sodium laurate at pH 7.5 and 1.25% normal serum albumin were analyzed by SDS-page electrophoresis under non-reducing conditions. Further, a sample of the purified IFN- β eluate from the G-25 pool that was unformulated was also tested as a control. The sodium laurate, Trycol® LAL(12)-Nopalcol®, Plurafac® C-17, and Trycol® LAL(12) did not look significantly different from the G-25 pool by SDS-page. There was an extra band present in the Triton® X305-Nopalcol® and Durfax® 80/SDS-page results. However, a Western blot was performed which showed that said band did not stain for IFN- β .

Example 7

Dextrose Lyophilized Formulations

The non-ionic detergent formulations listed in Table 1, as well as a 0.1% laurate formulation and a 1.25% human serum albumin formulation, were studied by ultracentrifugation after freeze-drying in 1.25% dextrose. Table III is a compilation of the ultracentrifugation data relating to the dextrose-formulated lyophilized samples. The ultracentrifugation procedure was as that described above in Example 5. Trycol® LAL(12) at 0.1% concentration and at pH 6.5 did not appear to resolubilize IFN- β after lyophilization. Higher concentrations of Trycol® LAL(12) were tested, and it was found that a concentration of 0.15% Trycol® was able to solubilize IFN- β at pH 7.0 with minor aggregation.

Since somewhat lower pH values can be tolerated clinically, Trycol® LAL(12) and Plurafac® C-17 were tested at pH 5.0 to see if lower levels of surfactants would solubilize IFN- β at this pH. UV scans show that both detergents were able to solubilize IFN- β at pH 5.0, at concentrations as low as 0.01%.

TABLE III

| Dextrose Lyophilized Formulations | | | |
|-----------------------------------|-------------------------|------------------------|------------|
| Sample | A ₂₈₀ Before | A ₂₈₀ After | % Recovery |
| Laurate | 1.023 | 0.485 | 47 |
| Triton® X305/Nopalcol | 1.325 | 0.960 | 72 |
| Trycol® LAL(12)/Nopalcol | 0.628 | 0.463 | 74 |
| Durfax® 80/SDS | 0.688 | 0.289 | 42 |
| Trycol® LAL(12) (0.1% pH 6.5) | 1.210 | 0.245 | 20 |
| Plurafac® C-17 (pH 6.0) | 0.561 | 0.394 | 70 |
| HSA Formulation | 1.469 | 0.814 | 55 |
| Starting pH 3.0 | 0.329 | 0.244 | 75 |

Example 8

Recombinant IFN- β in formulations with 0.15% Trycol® at pH 7.0 were tried with other bulking/stabilizing agents (non-reducing sugars) according to the formulation procedure of Example 1. However, in some cases, the formulations were not frozen, some were frozen, and some were frozen slowly. Ultracentrifugation was performed on such samples according to the procedure of Example 5. The test results are included as Table IV below. The results indicated that the different sugars had no effect on the solubility of IFN- β . However, the method of freezing the samples affected whether aggregates were formed. Freezing the samples quickly in dry ice and ethanol did not produce aggregates, while freezing slowing by placing the room-temperature samples in -20 °C caused some degree of aggregation.

TABLE IV

| Ultracentrifugation IFN- β in 0.15% Trycol®, pH 7.0, With Various Sugars at 5% | | | | | |
|--|-------------------------------------|---------------|-------------------------|------------------------|------------|
| | Samples | Treatment | A ₂₈₀ Before | A ₂₈₀ After | % Recovery |
| 1 | H ₂ O (no bulking agent) | None | 0.400 | 0.367 | 91.8 |
| 2 | Mannitol | None | 0.400 | 0.370 | 92.5 |
| 3 | Sorbitol | None | 0.409 | 0.378 | 92.4 |
| 4 | Dextrose | None | 0.496 | 0.484 | 97.6 |
| 5 | Inositol | None | 0.403 | 0.351 | 87.1 |
| 6 | H ₂ O | Frozen | 0.388 | 0.384 | 99.0 |
| 7 | Mannitol | Frozen | 0.412 | 0.379 | 92.0 |
| 8 | Sorbitol | Frozen | 0.404 | 0.384 | 95.1 |
| 9 | Dextrose | Frozen | 0.601 | 0.579 | 96.3 |
| 10 | Inositol | Frozen | 0.397 | 0.375 | 94.5 |
| 11 | Dextrose | Frozen Slowly | 0.656 | 0.584 | 89.0 |
| 12 | Mannitol | Frozen Slowly | 0.296 | 0.249 | 84.1 |

Example 9

Linear Non-Isothermal Stability Studies

Although linear, non-isothermal stability studies (LNS) cannot predict the absolute long-term stability of a given formulation, they are a useful tool in comparing the relative stability of different formulations and can be performed in a matter of days. Samples were prepared for the stability studies by chromatography on a G-25 desalting column using 0.1% sodium laurate as a transfer component, as described in Example 1. All the formulations tested for the LNS studies included 5% mannitol and were lyophilized. Final container vials were kept at 4°C until submitted for study. The linear non-isothermal stability (LNS) studies were performed running a linear temperature ramp from 50°C to 90°C at 1.5°C rise/hour. Bioactivity was measured by a yield reduction assay as described in Steward and Lockhart, *J. of Virol.*, 6:795-799 (1970).

At pre-set intervals, 0.5 ml was removed from each vial with sterile, plastic syringes, transferred to plastic assay tubes, and frozen at -20°C until assayed. The intervals were approximately 24 hours apart. The samples were thawed, randomized, and then submitted for yield reduction assay on either day 1, day 2, or day 3.

Figure 3 shows LNS study results for normal, high and low dose human serum albumin formulations of IFN- β as well as 1% sodium laurate formulations of IFN- β . The graph of Figure 3 indicates that the high dose HSA formulation is relatively more stable than the normal dose HSA formulation, which is, in turn, more stable than the low dose HSA and laurate formulations.

Figure 4 compares the relative stability of three mannitol formulations of the instant invention. The scale is the same as that for Figure 3. From a comparison of Figures 3 and 4, one would predict that the Triton X305/Nopalcol formulation is as stable as the normal dose HSA formulated IFN- β , and that Plurafac® C-17 formulation is as stable as the high dose HSA formulated IFN- β .

Figure 5 illustrates the relative stability of normal and high dose HSA formulated β -IFN and 0.25 mg/ml IFN- β in 5% mannitol with 0.1% Plurafac® C-17 at pH 6.0 as well as a 0.01% Plurafac® C-17 formulation at pH 5 as represented by the diamonds on Figure 5. The results show that the 0.1% Plurafac® C-17 formulation is at least as stable as the high-dose human serum albumin formulation. Further, the LNS study predicts that the 0.01% Plurafac® C-17 formulation at pH 5 will also have good long-term stability. The Trycol® LAL(12) at 0.15% formulation at pH 7 is predicted from the LNS study to be as stable as the high-dose HSA formulation, whereas the Trycol® LAL(12)-Nopalcol formulation should be more stable than the HSA normal dose formulation.

Example 10Toxicity Studies

5 IFN- β was purified as described in Example 1 wherein SDS was removed from the G-75 pool by desalting on a G-25 column run in 0.1% sodium laurate as a transfer component. Formulation with representative formulants of the invention was performed as described.

For these toxicity studies, 8 mice (4 female and 4 male) were tested per formulation. The representative formulations tested each contained one of the solubilizer/stabilizer compositions listed in Table I, *supra*, 5% mannitol and 0.25 mg/ml of IFN- β . All the formulations were lyophilized and reconstituted in sterile water. Each animal was injected with 0.2 ml intravenously via a lateral tail vein. Additionally, four mice (2 female and 2 male) were injected with 0.2 ml of excipient containing 1.0% detergent. These toxicity studies resulted in no deaths and no weight change of the tested mice. Such data confirm the oral toxicology reports submitted to applicants by the manufacturers of representative non-ionic surfactants used in the formulations of the invention as noted above in the summary of the invention.

Example 11Timing of Formulation Steps

20 The formulation step was performed the day after the desalting step employing sodium laurate as a transfer component on a G-25 column (see Example 1) was run and the sodium laurate was removed. The non-formulated IFN- β was kept at pH 3 and at 4 degrees centigrade overnight before formulation. Those formulations were compared to formulations made immediately after running the G-25 column. Results indicate that running the G-25 desalting column 24 hours before the formulation does not increase the level of aggregates.

When the G-25 column was run the day before, and IFN- β kept refrigerated over night at pH 3.0, and the formulations were neutralized to pH 7 four hours before lyophilization, a 30% to 40% increase in the amount of aggregation was obtained when compared to samples that were neutralized immediately before lyophilization. The results below in Table V indicate that lyophilization timing for representative formulations of the invention is critical to the level of aggregation. Lyophilization, immediately after neutralization of the IFN- β protein solution, results in very low levels of aggregates.

TABLE V

| LYOPHILIZATION TIMING | | |
|------------------------|--------|-------------|
| | 4 HRS. | IMMEDIATELY |
| TRITON® X305/NOPALCOL® | 58% | 92% |
| PLURAFAC® C-17 | 50% | 85% |

Example 12Mannitol Versus Dextrose Formulations

Table VI shows the results of ultracentrifugation studies on 5% mannitol, 2.5% mannitol and 5% dextrose formulations, respectively, wherein Trycol® 0.15% at pH 7 is the stabilizer/solubilizer. Ultracentrifugation data of such formulations prepared according to Example I indicate that mannitol in some way appears to interfere with the solubility of IFN- β . Therefore, dextrose alone is considered to be a more preferred bulking/stabilizing agent than mannitol alone for the formulations of this invention.

TABLE VI

| Ultracentrifugation Mannitol vs. Dextrose | |
|---|----------------------|
| | Trycol® 0.15% pH 7.0 |
| 5% Mannitol | 79.5% |
| 2.5% Mannitol | 84.5% |
| 5% Dextrose | 90.5% |

Example 13LNS Studies of Dextrose- and Mannitol-Containing Formulations

LNS studies were performed on representative formulations of the instant invention to compare dextrose versus mannitol as the bulking/stabilizing agent therein. Trycol® LAL(12) IFN- β compositions prepared as according to Example 1 were formulated with 5% dextrose and 5% mannitol, respectively. The LNS results are presented in Figure 6. A comparison with a normal and high dose HSA formulated IFN- β formulation indicate that the non-ionic surfactant containing formulations of the invention are predicted to be at least as stable as the normal dose HSA IFN- β formulation. No difference in stability was observed between the mannitol- and dextrose-formulated compositions.

Example 14

This example shows the results of a representative screening process of this invention for candidate formulating agents. G-75 purified IFN- β was eluted from a G-25 Sephadex column equilibrated in laurate buffer (phosphate) at pH 9.2. The sodium laurate was precipitated at pH 3 as described in Example 1. The soluble IFN- β was separated by centrifugation and added at pH 3 to the non-detergent stabilizer, glycerol, at a concentration by volume of 25%, and a non-ionic detergent, as indicated in Table VII below, at a concentration of 0.01%. The pH of the solutions was then raised to 5. The chromatography was performed at room temperature. After the formulations were made, they were observed for any formation of a precipitate; the observations were recorded; and then the samples were kept at 4 °C for 24 hours to several weeks.

All samples were centrifuged according to the procedures of Example 5, and the amount of IFN- β remaining in solution was calculated based on A_{280} . Aliquots for bioassay [by the cytopathic effect assay described by Steward, The Interferon System, p 17 (1981)] were taken at time = 0, 24 hours, 48 hours and one week.

The results of such experiments are shown in Table VII and VIII. Table VII indicates that 96-100% of IFN- β formulated with 25% glycerol and 0.01% of the specified non-ionic detergent remains soluble. Table VIII shows the bioassay results for such samples. After formulation, all the samples maintained bioactivity over the course of one week at 4 °C.

Table VII

| Recovery of Protein in the Supernatant for Various Formulations | | | |
|---|----------------------------|------------------------------------|----------------------------------|
| Formulation | Precipitation at 5 Minutes | % Recovery by A_{280} (24 Hours) | % Recovery by A_{280} (1 Week) |
| All Samples in 25% Glycerol and 25 mM NaPO ₄ | | | |
| 0.01% Trycol LAL-12, pH 5 | 0 | 100 | 96 |
| 0.01% Pluronic F-68, pH 5 | 0 | 97 | 102 |

Table VIII

| Specific Activity (CPE Assay) of Various Formulations (U/mg x 10 ⁷) | | | | |
|---|---------|----------|----------|--------|
| Sample | 0 Hours | 24 Hours | 48 Hours | 1 Week |
| 25% Glycerol, 0.01% Trycol®, pH 5 | 3.0 | 0.5 | 0.3 | 0.9 |
| 25% Glycerol, 0.01% Pluronic F-68, pH 5 | 10.0 | 9.7 | 9.7 | 4.1 |

Example 15

The example illustrates a number of different combinations of bulking/stabilizing agents for lyophilized formulations of IFN- β . The IFN- β used for this example was extracted and purified essentially according to Example 1, up to and including the point at which the IFN- β filtrate was stabilized by the addition of 0.15% Trycol® LAL-12. Then, 5% dextrose (wt./vol.) was added to a 10 ml aliquot of the IFN- β stabilized filtrate, and the following combinations of bulking/stabilizing agents were similarly each added respectively to 10 ml aliquots of the IFN- β filtrate (all percentages are weight to volume concentration ratios): 0.1% dextrose/2% mannitol; 0.2% dextrose/2% mannitol; and 0.1% dextrose/2% glycine. The pH of each 10 ml aliquot was then raised to about 6.0 with NaOH. The solutions were then pre-filtered and sterile filtered through 0.45 and 0.22 micron nitrocellulose filters, respectively. Then, the correct dosage amounts of the IFN- β_{ser17} (0.25 mg/ml) were aseptically filled into sterilized vials with sterilized stoppers under sanitary and sterile conditions that were carefully monitored.

The vials were then placed in a lyophilizer where appropriate thermocouples were attached. The lyophilization cycle proceeded as follows: the temperature was lowered to -30 °C at which temperature the vials were held for primary drying (under vacuum) for approximately 12 hours; and a two-stage ramp was performed wherein the first stage was a 3 °C/hour rise in temperature to 5 °C at which temperature the vials were held for 12 hours, and wherein the second stage was a 3 °C/hour rise in temperature to 15 °C at which temperature the vials were held for 24 hours. The vials were then refrigerated at 2 ° to 8 °C until they were reconstituted with water for injection, added in a 1/1 ratio (vol./vol.) and tested by ultracentrifugation and UV scanning as described in Example 5, above. The ultracentrifugation results are listed below in Table IX.

Table IX

| IFN- β Formulated with Trycol® LAL-12 and Various Bulking/Stabilizing Agents | |
|--|----------------------------------|
| Bulking/Stabilizing Reagents % (wt./vol.) | Ultracentrifugation Recovery (%) |
| 5 % Dextrose | 95% |
| 0.1% Dextrose/2% Mannitol | 88% |
| 0.2% Dextrose/2% Mannitol | 89% |
| 0.1% Dextrose/2% Glycine | 76% |

The plug appearance for each, formulation was good. If a standard lyophilization procedure were performed, for example, a primary drying period at -30 °C for 12 hours followed by a 12 °C/hour ramp to 15 °C, the plug for the 5% dextrose containing formulation appears somewhat collapsed, although the ultracentrifugation recovery and UV scan results are equivalent to that achieved with the above-described slow, two-stage ramp lyophilization protocol. However, the plug appearance for the formulations, other than those containing 5% dextrose as the bulking stabilizing agent, lyophilized according to a standard protocol is the same as for that when a two-stage ramping protocol is employed.

The UV scanning results showed very little aggregation for the formulations containing 5% dextrose, 0.1% dextrose/2% mannitol, and 0.2% dextrose/2% mannitol, as bulking/stabilizing agents; however, more aggregation was seen in the formulation containing 0.1% dextrose/2% glycine as the bulking/stabilizing agent, making it a less preferred bulking/stabilizing agent combination than the others tested.

Conclusion

In summary, it can be seen that the IFN- β formulations of the present invention containing biocompatible non-ionic polymeric detergents screened according to the processes of the instant invention and a bulking/stabilizing agent are desirable pharmaceutical compositions. Said recombinant IFN- β formulations are at least as stable as HSA formulations, and do not contain significant amounts of aggregates. Improved formulation processes are also described herein which result in formulations having very low level of aggregates and other potentially immunogenic characteristics and minimal or no amounts of strong solubilizing agents, such as SDS. The formulations of this invention are further non-toxic and have good shelf life.

Deposits

As mentioned above, a culture of *E. coli* K12/MM294-1 carrying plasmid pSY2501 was deposited at the American Type Culture Collection 12301 Parklawn Drive, Rockville, MD 20852, US, on November 18, 1983 under ATCC No. 39,517.

Said deposit was made pursuant to a contract between the ATCC and the assignee of this patent application, Cetus Corporation. The contract with the ATCC provides for permanent availability of said strain and progeny thereof to the public upon issuance of a U.S. patent related to this application describing and identifying the deposit or upon the publication or laying open to the public of any U.S. or foreign patent application, whichever comes first, and for the availability of the strain and the progeny thereof to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the strain on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced upon notification with a viable culture of the same strain.

The deposit under the terms of the Budapest Treaty assure that said culture deposited will be maintained in a viable and uncontaminated condition for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism was received by the ATCC and, in any case, for a period of at least 30 years after the date of the deposit.

The culture was made available on May 21, 1985. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Also, the present invention is not to be considered limited in scope by the strain deposited, since the deposited embodiments are intended only to be illustrative of particular aspects of the invention. Any microorganism strain which is functionally equivalent to those deposited are considered to be within the scope of this invention.

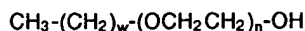
Claims

1. A stable pharmaceutical composition of matter suitable for parenteral administration to animals or humans comprising a therapeutically-effective amount of an unglycosylated, recombinant interferon- β protein dissolved in an inert, aqueous-based carrier medium comprising, as a solubilizer/stabilizer, an effective amount of one or more biocompatible non-ionic polymeric detergents or solubilizers/stabilizers, wherein said detergent is an ethoxylated fatty alcohol ether and lauryl ether; an octylphenoxy polyethoxy ethanol compound; a modified oxyethylated or oxypropylated straight-chain alcohol or a mixture of both; a polyethylene glycol monooleate compound; a phenolic fatty alcohol ether; or a block copolymer of propylene oxide and ethylene oxide wherein said composition additionally contains SDS or glycerol when said detergent is only a block copolymer of propylene oxide and ethylene oxide.
2. A composition according to claim 1 wherein said carrier medium further comprises an additional solubilizing or stabilizing agent.
3. A composition according to claim 1 or 2 which is either in liquid form or lyophilized.
4. A composition according to claim 3 which is in liquid form and wherein said additional solubilizing or stabilizing agent is glycerol in a concentration (v/v) range of from 5% to 50% and said biocompatible

non-ionic detergent is in a concentration (v/v) range of from 0.0005% to 5%.

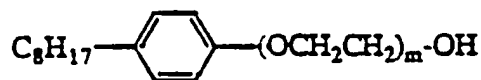
5. A composition according to any one of claims 1-4 wherein said detergent is:

(a) a mixture of ethoxylated fatty alcohol ethers and lauryl ether compounds having the formula:



wherein n is a distribution from 1 to 30 and centering on $n = 12$, and w is a distribution from 9 to 17, containing a significant portion of $w = 11$;

(b) an octylphenoxy polyethoxy ethanol compound having the formula:



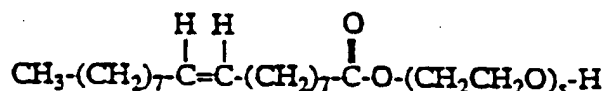
wherein m is an integer between 5 and 60;

(c) a mixture of modified oxyethylated or oxypropylated straight-chain alcohols or of both having the formula:



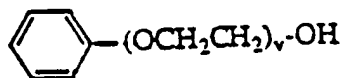
wherein p is an integer between 1 and 10; q is zero or an integer between 1 and 10; and r is an integer between 6 and 14;

(d) a polyethylene glycol monooleate compound having the formula:



wherein s is an integer between 1 and 10;

(e) a phenolic fatty alcohol ether having the formula



wherein v is an integer between 10 and 200; or

(f) block copolymers of propylene oxide and ethylene oxide (Pluronic® polyols).

6. A composition according to claim 5 wherein said detergent is:

(a) polyoxyethylene 12 lauryl ether mixture which is an ethoxylated fatty alcohol ether, polyoxyethylene (12) lauryl ether (Trycol® LAL-12); a polyoxyethylene (12) lauryl alcohol (Macol® LA-12) or a polyoxyethylene (12) lauryl alcohol (Siponic® L-12);

(b) an octylphenoxy polyethoxy ethanol compound wherein m is an integer between 20 and 50;

(c) a mixture of modified oxyethylated or oxypropylated straight-chain alcohol compound or of both;

wherein p is an integer between 1 and 5;

q is zero or an integer between 1 and 5; and

r is an integer between 10 and 12;

(d) a polyethylene glycol monooleate compound wherein s is an integer between 2 and 6;

(e) a phenolic fatty alcohol ether wherein v is an integer between 50 and 150; or

(f) a block copolymer of propylene oxide and ethylene oxide.

7. A composition according to claim 6 wherein said detergent is

(a) a polyoxyethylene (12) lauryl alcohol (Macol® LA-12);

- (b) an octylphenoxy polyethoxy ethanol compound wherein m is 30 or 40;
- (c) a modified oxyethylated straight-chain alcohol (Plurafac® C17);
- (d) a polyethylene glycol ther wherein s is 3; and
- (e) a phenolic fatty alcohol ther wherein v is 100.

5

8. A composition according to claim 7 wherein said detergent is:

- (a) a polyoxyethylene (12) lauryl-alcohol (Macol® LA-12);
- (b) an octylphenoxy polyethoxy ethanol (Triton® X305);
- (c) a modified oxyethylated straight-chain alcohol (Plurafac® C-17);
- (d) a polyethylene glycol 400 mono-oleate (Nopalcol® 4-0).

10

9. A composition according to any one of claims 1-8 wherein the concentration (v/v) range of said nonionic detergent is from 0.005% to 5%.

15

10. A composition according to claim 3 which is in liquid form and further comprises one or more carbohydrate or non-carbohydrate stabilizing agents in a concentration (v/v) range of from 0.025% to 10%.

20

11. A composition according to claim 10 wherein said carbohydrate stabilizing agents are sucrose, fructose, dextrose, dextran, mannitol, sorbitol, inositol, galactitol, xylitol, lactose or trehalose and said non-carbohydrate stabilizing agent is human serum albumin.

25

12. A composition according to claim 3 which is lyophilized and reconstitutable and wherein said inert carrier medium further comprises a buffer and a bulking/stabilizing agent wherein said buffer is at a concentration range of from 1 to 50 mM and is acetate or phosphate; and said bulking/stabilizing agent is dextrose alone at a concentration (v/v) range of from 1% to 10%; a combination of dextrose at a concentration (v/v) range of from 0.1% to 5% with mannitol at a concentration (v/v) range of from 0.5% to 5%; a combination of dextrose at a concentration (v/v) range from 0.1% to 5% with human serum albumin at a concentration (v/v) range of from 1% to 5%; or human serum albumin alone at a concentration (v/v) range of from 1% to 5%.

30

13. A composition according to claim 12 wherein said buffer is phosphate at a concentration from 10 mM to 25 mM, and said bulking/stabilizing agent is dextrose at a concentration (v/v) of 5% or a combination of dextrose and mannitol at a concentration ratio of from 1/5 to 1/20 of dextrose/mannitol.

35

14. A composition according to claim 12 or 13 wherein the non-ionic detergent is polyoxyethylene 12 lauryl ether at a concentration (v/v) of from 0.05% to 5%; the bulking/stabilizing agent is a combination of dextrose at a concentration (v/v) from 0.1% to 0.2% and mannitol at a concentration (v/v) from 1% to 3%; the buffer is phosphate at a concentration range of 15 mM to 25 mM; the IFN- β is at a concentration from 0.125 to 2 mg/ml; and the pH of the composition ranges from 3.5 to about 9.5.

40

15. A composition according to claim 14 wherein the non-ionic detergent is a polyoxyethylene (12) lauryl alcohol (Macol® LA-12) at a concentration (v/v) of 0.15%; the bulking/stabilizing agent is a combination of dextrose at a concentration (v/v) of 0.2% and mannitol at a concentration (v/v) of 2%; the phosphate buffer is at a concentration of 20 mM; IFN- β is at a concentration of 0.25 mg/ml; and the pH of the composition is 6.

45

16. A composition according to any one of claims 1-15 wherein the detergents are at respective concentrations (v/v) indicated by percentages as follows: 0.1% polyoxyethylene 12 lauryl ether and 0.05% polyethylene glycol 400 monooleate (Nopalcol® 4-0); 0.1% octylphenoxy polyethoxy ethanol (Triton® X305); 0.05% polyethylene glycol 400 monooleate (Nopalcol® 4-0); and 0.1% octylphenoxy polyethoxy ethanol (Triton® X405), 0.05% polyethylene glycol 400 monooleate (Nopalcol® 4-0); and the IFN- β is at a concentration of 0.25 mg/ml.

50

55

17. A composition according to any one of claims 1-16 wherein the recombinant interferon- β is IFN- β_{ser17} .

18. A composition according to any one of claims 1-17 wherein said IFN- β is maintained in solution at a pH of 3.5 to 9.5 over a 24-hour period at 4 °C.

19. A composition according to claim 18 wherein the pH is neutral pH.
20. A composition according to claim 3 which is lyophilized and reconstitutable and where in said inert carrier medium further comprises: a buffer; or a bulking/stabilizing agent and a buffer; said buffer concentration is between 1 and 50 mM and is acetate or phosphate; and said bulking/stabilizing agent is a combination of between 0.1% and 5% sorbitol and between 0.1% and 5% mannitol.
21. A composition according to claim 20 wherein the non-ionic detergent is polyoxyethylene 12 lauryl ether at a concentration (v/v) between 0.05% and 5%; the buffer is phosphate at a concentration range between 15 mM and 25 mM; the IFN- β is at a concentration between 0.125 and 2 mg/ml; and the pH is between 3.5 and 9.5.
22. A composition according to claim 21 wherein the non-ionic detergent is a polyoxyethylene (12) lauryl alcohol (Macol® LA-2) at a concentration (v/v) of 0.15%; the phosphate buffer is at a concentration of 20 mM; the IFN- β is at a concentration of 0.25 mg/ml; and the pH of the composition is 6.
23. A composition according to claim 1, further comprising a pharmaceutically acceptable excipient whereby the composition is rendered orally administrable.
24. A method of screening for one or more biocompatible non-ionic polymeric detergents or for combinations of one or more of said detergents with another solubilizing or stabilizing agent capable of solubilizing and stabilizing pharmaceutical compositions of unglycosylated recombinant interferon- β (IFN- β), comprising steps of:
- (a) passing extracted, purified IFN- β in sodium dodecyl sulfate (SDS) on a desalting column equilibrated in sodium laurate in an elution buffer at pH 9.0-10.0 to form an eluate;
 - (b) lowering the pH of the eluate to about pH 2-3.3;
 - (c) centrifuging and filtering the eluate to remove the precipitated sodium laurate;
 - (d) adding to the filtrate an effective amount of one or more of said detergents or said combination of one or more of said detergents with another solubilizing or stabilizing agent;
 - (e) adjusting the pH of the filtrate to 3.5 to 9.5;
 - (f) allowing said filtrate to stand for about 25 hours at pH 3.5 to 9.5; and
 - (g) determining whether the recombinant IFN- β was maintained in solution.
25. A method according to claim 24 wherein said other solubilizing or stabilizing agent is glycerol at a concentration (v/v) range of from about 5% to about 50%.
26. A method of preparing stable, pharmaceutical compositions of unglycosylated recombinant interferon- β (IFN- β) protein comprising the steps of:
- (a) extracting the IFN- β from the disruptate of a host organism transformed to produce the protein;
 - (b) purifying the IFN- β using as the last purification step a desalting step at a pH range of 8.5 to 10 employing an elution buffer containing a fatty acid salt having a carbon chain containing from 10 to 12 carbons to form a desalted pool;
 - (c) lowering the pH of the desalted pool to a pH of 2 to 4 thereby precipitating the fatty acid salt;
 - (d) removing the precipitated salt from the pool by centrifugation and filtration;
 - (e) adding to the desalted pool an effective amount of one or more non-ionic biocompatible polymeric detergents, or a combination of one or more biocompatible non-ionic polymeric detergents and another solubilizing or stabilizing agent, in effective amounts to stabilize and solubilize the IFN- β wherein said detergent is an ethoxylated fatty alcohol ether and lauryl ether, an octylphenoxy polyethoxy ethanol compound; a modified oxyethylated or oxypropylated straight-chain alcohol or a mixture of both; a polyethylene glycol monooleate compound; a phenolic fatty alcohol ether; or a block copolymer of propylene oxide and ethylene oxide wherein said composition additionally contains SDS or glycerol when said detergent is only a block copolymer of propylene oxide and ethylene oxide;
 - (f) adjusting the pH of the pool to a range of between 4.0 and 8.0;
 - (g) adding an effective amount of a bulking/stabilizing agent to the pool; and
 - (h) lyophilizing the formulation.

27. A method of preparing stable, pharmaceutical compositions of unglycosylated recombinant interferon- β (IFN- β) protein comprising the steps of:
- (a) isolating refractile bodies containing the IFN- β from a host organism transformed to produce said IFN- β ;
 - 5 (b) solubilizing said refractile bodies by employing sodium laurate;
 - (c) extracting and purifying said IFN- β from the solubilized refractile material employing sodium laurate as the primary solubilizing agent;
 - (d) lowering the pH of the purified IFN- β to a pH from 2 to 4;
 - 10 (e) centrifuging and filtering the purified IFN- β solution to remove the sodium laurate precipitate to create an IFN- β pool;
 - (f) desalting the IFN- β pool at a pH from 2 to 4;
 - (g) adding to the IFN- β protein solution an effective amount of one or more non-ionic biocompatible polymeric detergents, or of a combination of a non-ionic biocompatible polymeric detergent and another solubilizing or stabilizing agent to stabilize and solubilize the IFN- β wherein said detergent is
 - 15 an ethoxylated fatty alcohol ether and lauryl ether, an octylphenoxy polyethoxy ethanol compound; a modified oxyethylated or oxypropylated straight-chain alcohol or a mixture of both; a polyethylene glycol monooleate compound; a phenolic fatty alcohol ether; or a block copolymer of propylene oxide and ethylene oxide wherein said composition additionally contains SDS or glycerol when said detergent is only a block copolymer of propylene oxide and ethylene oxide;
 - 20 (h) adjusting the pH of the IFN- β solution to a pH range of 3.5 to 9.5;
 - (i) adding to the solution an effective amount of a bulking/stabilizing agent; and
 - (j) lyophilizing the formulation.
28. A method of extracting unglycosylated recombinant interferon- β (IFN- β) from a bacterial host transformed to produce it and then purifying and formulating said IFN- β comprising the steps of:
- 25 (a) growing the transformed hosts in an appropriate fermentation medium;
 - (b) concentrating the bacterial host in the fermentation medium;
 - (c) disrupting the cell wall and cell membrane of the bacterial host;
 - 30 (d) removing greater than 99% by weight of the salts from said disruptate by diafiltration or centrifugation;
 - (e) redispersing the desalted disruptate;
 - (f) adding a material to the disruptate to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate;
 - 35 (g) separating the refractile material from the cellular debris by high-speed centrifugation;
 - (h) solubilizing the refractile material in an aqueous buffer containing a reducing agent;
 - (i) extracting the solubilized refractile material with 2-butanol or 2-methyl-2-butanol;
 - (j) isolating said refractile material from the extractant;
 - (k) solubilizing the resulting IFN- β particle pellet with an aqueous solution of sodium dodecyl sulfate at an IFN- β to sodium dodecyl sulfate ratio of 1:3 to form a solution;
 - 40 (l) adjusting the pH of the solution to 9.5 and reducing the solubilized IFN- β with dithiothreitol;
 - (m) purifying the reduced IFN- β by chromatography;
 - (n) oxidizing the IFN- β from step (m);
 - (o) further purifying the oxidized IFN- β by gel chromatography and collecting the eluate containing the purified IFN- β ;
 - 45 (p) desalting the purified IFN- β eluate in a desalting column equilibrated and run in sodium laurate at pH 9.0 to 9.8;
 - (q) lowering the pH of the eluate to pH 3.0;
 - (r) centrifuging and filtering the solution to remove the precipitate;
 - 50 (s) adding to the filtrate an effective amount of one or more non-ionic biocompatible polymeric detergents or a combination of one or more of said detergents and another solubilizing or stabilizing agent, to solubilize and stabilize the IFN- β ; wherein said detergent is an ethoxylated fatty alcohol ether and lauryl ether, an octylphenoxy polyethoxy ethanol compound; a modified oxyethylated or oxypropylated straight-chain alcohol or a mixture of both; a polyethylene glycol monooleate compound; a phenolic fatty alcohol ether; or a block copolymer of propylene oxide and ethylene oxide
 - 55 wherein said composition additionally contains SDS or glycerol when said detergent is only a block copolymer of propylene oxide and ethylene oxide,
 - (t) adjusting the pH of the solution to 4.0 to 9.5;
 - (u) adding an appropriate bulking/stabilizing agent in a concentration of from 0.25% to 10%; and

(v) lyophilizing the IFN- β solution.

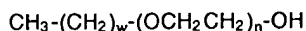
29. A method according to claim 28 wherein the bulking/stabilizing agent of step (u) is dextrose and wherein the lyophilizing of step (v) is performed by a procedure incorporating a two stage ramp.

30. A method according to claim 29 wherein the lyophilization procedure comprises the steps of:

- (a) freezing the IFN- β solution for from about 2 to about 6 hours at a temperature range from -70 ° to -20 ° C;
- (b) lyophilizing the frozen IFN- β solution of step (a) for from 12 to 48 hours;
- (c) ramping at a rate of from 3 ° C/hour to 12 ° C/hour to a temperature range from -5 ° to +10 ° C;
- (d) holding at the temperature range of step (c) for from 6 to 18 hours;
- (e) ramping for from 3 ° C/hour to 12 ° C/hour to a temperature range of +10 ° C to +25 ° C; and
- (f) holding at the temperature range of step (e) for at least 12 hours.

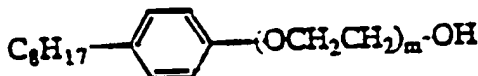
Patentansprüche

1. Stabiles Arzneimittel, das für die parenterale Verabreichung an Tiere oder Menschen geeignet ist, umfassend eine therapeutisch wirksame Menge eines nicht-glykosylierten rekombinanten Interferon- β -Proteins, gelöst in einem inerten Trägermedium auf Wasserbasis, umfassend als Solubilisierungsmittel/Stabilisierungsmittel eine wirksame Menge eines oder mehrerer biokompatiblen nichtionischen polymeren Detergentien oder Solubilisierungsmittel/Stabilisierungsmittel, wobei das Detergens ein ethoxylierter Fettalkoholether und Laurylether, eine Octylphenoxypolyethoxyethanolverbindung, ein modifizierter oxyethylierter oder oxypropylierter geradkettiger Alkohol oder ein Gemisch von beiden, eine Polyethylenglykolmonooleatverbindung, ein phenolischer Fettalkoholether oder ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist, wobei das Mittel zusätzlich SDS oder Glycerin enthält, wenn das Detergens nur ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist.
2. Mittel nach Anspruch 1, wobei das Trägermedium außerdem ein zusätzliches Solubilisierungs- oder Stabilisierungsmittel enthält.
3. Mittel nach Anspruch 1 oder 2, das entweder in flüssiger oder gefriergetrockneter Form vorliegt.
4. Mittel nach Anspruch 3, das in flüssiger Form vorliegt und wobei das zusätzliche Solubilisierungs- oder Stabilisierungsmittel Glycerin in einem Konzentrationsbereich (v/v) von 5 bis 50 % ist und das biokompatible nichtionische Detergens in einem Konzentrationsbereich (v/v) von 0,0005 % bis 5 % vorliegt.
5. Mittel nach einem der Ansprüche 1 bis 4, wobei das Detergens folgende Zusammensetzung hat:
 - (a) ein Gemisch aus ethoxylierten Fettalkoholether- und Lauryletherverbindungen mit der Formel:



in der n eine Verteilung von 1 bis 30 ist und hauptsächlich n = 12 ist, und w eine Verteilung von 9 bis 17 bedeutet, enthaltend einen wesentlichen Anteil von w = 11,

(b) eine Octylphenoxypolyethoxyethanolverbindung mit der Formel:



in der m eine ganze Zahl zwischen 5 und 60 ist,

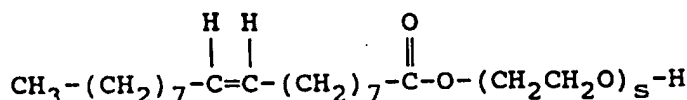
(c) ein Gemisch von modifizierten oxyethylierten oder oxypropylierten geradkettigen Alkoholen oder von beiden mit der Formel



in der p eine ganze Zahl zwischen 1 und 10 ist, q 0 ist oder eine ganze Zahl zwischen 1 und 10 und

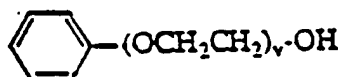
r eine ganze Zahl zwischen 6 und 14 ist,

(d) eine Polyethylenglykolmonooleatverbindung mit der Formel



in der s eine ganze Zahl zwischen 1 und 10 ist,

(e) ein phenolischer Fettalkoholether mit der Formel



in der v eine ganze Zahl zwischen 10 und 200 ist, oder

(f) Blockcopolymere aus Propylenoxid und Ethylenoxid (Pluronic®-Polyole).

6. Mittel nach Anspruch 5, wobei das Detergens die folgende Zusammensetzung hat:

(a) Polyoxyethylen-12-laurylether-Gemisch, das ein ethoxylierter Fettalkoholether, Polyoxyethylen-(12)-laurylether (Trycol® LAL-12), ein Polyoxyethylen-(12)-laurylalkohol (Macol® LA-12) oder ein Polyoxyethylen-(12)-laurylalkohol (Siponic® L-12) ist,

(b) eine Octylphenoxypolyethoxyethanolverbindung, in der m eine ganze Zahl zwischen 20 und 50 ist,

(c) ein Gemisch aus einer modifizierten oxyethylierten oder oxypropylierten geradkettigen Alkoholverbindung oder beiden,

wobei p eine ganze Zahl zwischen 1 und 5 ist,

q 0 ist oder eine ganze Zahl zwischen 1 und 5 und

r eine ganze Zahl zwischen 10 und 12 ist,

(d) eine Polyethylenglykolmonooleatverbindung, in der s eine ganze Zahl zwischen 2 und 6 ist,

(e) ein phenolischer Fettalkoholether, wobei v eine ganze Zahl zwischen 50 und 150 ist, oder

(f) ein Blockcopolymer aus Propylenoxid und Ethylenoxid.

7. Mittel nach Anspruch 6, wobei das Detergens die folgende Zusammensetzung hat:

(a) ein Polyoxyethylen-(12)-laurylalkohol (Macol® LA-12),

(b) eine Octylphenoxypolyethoxyethanolverbindung, in der m 30 oder 40 ist,

(c) ein modifizierter oxyethylierter geradkettiger Alkohol (Plurafac® C17),

(d) ein Polyethylenglykolether, in dem s den Wert 3 hat, und

(e) ein phenolischer Fettalkoholether, in dem v den Wert 100 hat.

8. Mittel nach Anspruch 7, wobei das Detergens die folgende Zusammensetzung hat:

(a) ein Polyoxyethylen-(12)-laurylalkohol (Macol® LA-12),

(b) ein Octylphenoxypolyethoxyethanol (Triton® X305),

(c) ein modifizierter oxyethylierter geradkettiger Alkohol (Plurafac® C-17),

(d) ein Polyethylenglykol-400-monooleat (Nopalcol® 4-0).

9. Mittel nach einem der Ansprüche 1 bis 8, wobei der Konzentrationsbereich (v/v) des nicht-ionischen Detergens 0,005 % bis 5 % beträgt.

10. Mittel nach Anspruch 3, das in flüssiger Form vorliegt und außerdem ein oder mehrere Kohlenhydrat- oder Nichtkohlenhydrat-Stabilisierungsmittel in einem Konzentrationsbereich (v/v) von 0,025 % bis 10 % umfaßt.

11. Mittel nach Anspruch 10, wobei das Kohlenhydrat-Stabilisierungsmittel Saccharose, Fructose, Dextrose, Dextran, Mannit, Sorbit, Inosit, Galactit, Xylit, Lactose oder Trehalose ist und das Nichtkohlenhydrat-

Stabilisierungsmittel menschliches Serumalbumin ist.

12. Mittel nach Anspruch 3, das gefriergetrocknet und wiederauflösbar ist und wobei das inerte Trägermedium außerdem einen Puffer und ein Füll-/Stabilisierungsmittel umfaßt, wobei der Puffer in einem Konzentrationsbereich von 1 bis 50 mM vorliegt und Acetat oder Phosphat ist, und das Füll-/Stabilisierungsmittel Dextrose allein in einem Konzentrationsbereich (v/v) von 1 % bis 10 % vorliegt, eine Kombination von Dextrose in einem Konzentrationsbereich (v/v) von 0,1 % bis 5 % mit Mannit in einem Konzentrationsbereich (v/v) von 0,5 % bis 5 %, einer Kombination von Dextrose in einem Konzentrationsbereich (v/v) von 0,1 % bis 5 % mit menschlichem Serumalbumin in einem Konzentrationsbereich (v/v) von 1 % bis 5 % oder menschliches Serumalbumin allein in einem Konzentrationsbereich (v/v) von 1 % bis 5 % ist.
13. Mittel nach Anspruch 12, wobei der Puffer Phosphat in einer Konzentration von 10 mM bis 25 mM und das Füll-/Stabilisierungsmittel Dextrose in einer Konzentration (v/v) von 5 % oder eine Kombination von Dextrose und Mannit in einem Konzentrationsverhältnis von 1/5 bis 1/20 von Dextrose/Mannit ist.
14. Mittel nach Anspruch 12 oder 13, wobei das nicht-ionische Detergens Polyoxyethylen-12-lauryl ether in einer Konzentration (v/v) von 0,05 % bis 5 %, das Füll-/Stabilisierungsmittel eine Kombination von Dextrose in einer Konzentration (v/v) von 0,1 % bis 0,2 % und Mannit in einer Konzentration (v/v) von 1 % bis 3 %, der Puffer Phosphat in einem Konzentrationsbereich von 15 mM bis 25 mM ist, das IFN- β in einer Konzentration von 0,125 bis 2 mg/ml vorliegt und der pH-Wert des Mittels im Bereich von 3,5 bis etwa 9,5 liegt.
15. Mittel nach Anspruch 14, wobei das nicht-ionische Detergens ein Polyoxyethylen-(12)-laurylalkohol (Macol® LA-12) in einer Konzentration (v/v) von 0,15 %, das Füll-/Stabilisierungsmittel eine Kombination von Dextrose in einer Konzentration (v/v) von 0,2 % und Mannit in einer Konzentration (v/v) von 2 % ist, der Phosphatpuffer in einer Konzentration von 20 mM, IFN- β in einer Konzentration von 0,25 mg/ml vorliegt und der pH-Wert des Mittels 6 beträgt.
16. Mittel nach einem der Ansprüche 1 bis 15, wobei die Detergentien in den jeweiligen Konzentrationen (v/v) vorliegen, die in Prozent wie folgt angegeben sind: 0,1 % Polyoxyethylen-12-lauryl ether und 0,05 % Polyethylenglykol-400-monooleat (Nopalcol® 4-0), 0,1 % Octylphenoxypolyethoxyethanol (Triton® X305), 0,05 % Polyethylenglykol-400-monooleat (Nopalcol® 4-0) und 0,1 % Octylphenoxypolyethoxyethanol (Triton® X405), 0,05 % Polyethylenglykol-400-monooleat (Nopalcol® 4-0) und das IFN- β in einer Konzentration von 0,25 mg/ml vorliegt.
17. Mittel nach einem der Ansprüche 1 bis 16, wobei das rekombinante Interferon- β IFN- β_{ser17} ist.
18. Mittel nach einem der Ansprüche 1 bis 17, wobei das IFN- β in einer Lösung bei einem pH-wert von 3,5 bis 9,5 über einen Zeitraum von 24 Stunden bei 4 °C gehalten wird.
19. Mittel nach Anspruch 18, wobei der pH-Wert ein neutraler pH-Wert ist.
20. Mittel nach Anspruch 3, das gefriergetrocknet und wiederauflösbar ist, und wobei das inerte Trägermedium weitere folgende Bestandteile hat: einen Puffer oder ein Füll-/Stabilisierungsmittel und einen Puffer, wobei die Pufferkonzentration zwischen 1 und 50 mM liegt, und der Puffer Acetat oder Phosphat ist, und das Füll-/Stabilisierungsmittel eine Kombination aus zwischen 0,1 % und 5 % Sorbit und zwischen 0,1 % und 5 % Mannit ist.
21. Mittel nach Anspruch 20, wobei das nicht-ionische Detergens Polyoxyethylen-12-lauryl ether in einer Konzentration (v/v) zwischen 0,05 % und 5 % ist, der Puffer Phosphatpuffer in einem Konzentrationsbereich zwischen 15 mM und 25 mM ist, das IFN- β in einer Konzentration zwischen 0,125 und 2 mg/ml vorliegt und der pH-Wert zwischen 3,5 und 9,5 liegt.
22. Mittel nach Anspruch 21, wobei das nicht-ionische Detergens ein Polyoxyethylen-(12)-laurylalkohol (Macol® LA-12) in einer Konzentration (v/v) von 0,15 % ist, der Phosphatpuffer in einer Konzentration von 20 mM, das IFN- β in einer Konzentration von 0,25 mg/ml vorliegt und der pH-Wert des Mittels 6 ist.

23. Mittel nach Anspruch 1, außerdem einen pharmazeutisch verträglichen Excipienten umfassend, wobei das Mittel oral verabreichbar ist.
24. Verfahren zum Absuchen auf ein s oder mehrere biokompatible nicht-ionische polymere Detergentien oder auf Kombinationen von einem oder mehreren dieser Detergentien mit einem anderen Solubilisierungs- oder Stabilisierungsmittel, das Arzneimittel mit unglykosyliertem rekombinante Interferon- β (IFN- β) solubilisieren und stabilisieren kann, umfassend die folgenden Schritte:
- Durchleiten von extrahiertem gereinigtem IFN- β in Natriumdodecylsulfat (SDS) durch eine Entsalzersäule, die mit Natriumlaurat in einem Elutionspuffer bei einem pH-Wert von 9,0 bis 10,0 äquilibriert ist, wobei ein Eluat erhalten wird,
 - Erniedrigung des pH-Werts des Eluats auf etwa 2 bis 3,3,
 - Zentrifugation und Filtration des Eluats zur Entfernung des ausgefallenen Natriumlaurats,
 - Zugabe einer wirksamen Menge eines oder mehrerer der Detergentien oder der Kombination von einem oder mehreren der Detergentien mit einem anderen Solubilisierungs- oder Stabilisierungsmittel zum Filtrat,
 - Einstellung des pH-Wertes des Filtrats auf 3,5 bis 9,5,
 - Stehenlassen des Filtrats für etwa 25 Stunden bei einem pH-Wert von 3,5 bis 9,5 und
 - Bestimmung, ob das rekombinante IFN- β in Lösung geblieben ist.
25. Verfahren nach Anspruch 24, wobei das andere Solubilisierungs- oder Stabilisierungsmittel Glycerin in einem Konzentrationsbereich (v/v) von etwa 5 % bis etwa 50 % ist.
26. Verfahren zur Herstellung von stabilen Arzneimitteln aus unglykosyliertem rekombinante Interferon- β (IFN- β)-Protein, umfassend die folgenden Schritte:
- Extraktion des IFN- β aus den aufgebrochenen Zellen eines Wirtsorganismus, der zur Produktion des Proteins transformiert wurde,
 - Reinigung des IFN- β , wobei als letzter Reinigungsschritt ein Entsalzungsschritt bei einem pH-Wert von 8,5 bis 10 durchgeführt wird, wobei ein Elutionspuffer eingesetzt wird, der ein Fettsäuresalz mit einer Kohlenstoffkette mit 10 bis 12 Kohlenstoffatomen enthält, wobei ein entsalzter Pool erhalten wird,
 - Erniedrigen des pH-Werts des entsalzten Pools auf einen Wert von 2 bis 4, wobei das Fettsäuresalz ausfällt,
 - Entfernung des ausgefallenen Salzes aus dem Pool durch Zentrifugation und Filtration,
 - Zugabe einer wirksamen Menge eines oder mehrerer nicht-ionischer biokompatibler polymerer Detergentien oder einer Kombination von einem oder mehreren biokompatiblen, nicht-ionischen polymeren Detergentien und einem anderen Solubilisierungs- oder Stabilisierungsmittel in wirksamen Mengen, so daß das IFN- β stabilisiert und solubilisiert wird, zu dem entsalzten Pool, wobei das Detergens ein ethoxylierter Fettalkoholether und Laurylether, eine Octylphenoxypolyethoxyethanolverbindung, ein modifizierter oxyethylierter oder oxypropylierter geradkettiger Alkohol oder ein Gemisch von beiden, eine Polyethylenglykolmonooleatverbindung, ein phenolischer Fettalkoholether oder ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist, wobei das Mittel zusätzlich SDS oder Glycerin enthält, wenn das Detergens nur ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist,
 - Einstellung des pH-Werts des Pools auf einen Bereich zwischen 4,0 und 8,0,
 - Zugabe einer wirksamen Menge eines Füll-/Stabilisierungsmittels zum Pool, und
 - Gefriertrocknung der Formulierung.
27. Verfahren zur Herstellung von stabilen Arzneimitteln aus unglykosyliertem rekombinante Interferon- β (IFN- β)-Protein, umfassend die folgenden Schritte:
- Isolierung von das IFN- β enthaltenden stark lichtbrechenden Körperchen aus einem Wirtsorganismus, der zur Produktion des IFN- β transformiert wurde,
 - Solubilisierung der stark lichtbrechenden Körperchen unter Verwendung von Natriumlaurat,
 - Extraktion und Reinigung des IFN- β aus dem solubilisierten stark lichtbrechenden Material unter Verwendung von Natriumlaurat als primäres Solubilisierungsmittel,
 - Erniedrigen des pH-Werts des gereinigten IFN- β auf einen Wert von 2 bis 4,
 - Zentrifugation und Filtration der gereinigten IFN- β -Lösung zur Entfernung des Natriumlauratniederschlags, wobei ein IFN- β -Pool erhalten wird,
 - Entsalzen des IFN- β -Pools bei einem pH-Wert von 2 bis 4,

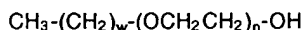
- (g) Zugabe einer wirksamen Menge eines oder mehrerer nicht-ionischer biokompatibler polymerer Detergentien oder einer Kombination aus einem nicht-ionischen biokompatiblen polymeren Detergens und einem anderen Solubilisierungs- oder Stabilisierungsmittel zu der IFN- β -Proteinlösung zur Stabilisierung und Solubilisierung des IFN- β , wobei das Detergens ein ethoxylierter Fettalkoholether und Laurylether, eine Octylphenoxypolyethoxyethanolverbindung, ein modifizierter oxyethylierter oder oxypropylierter geradkettiger Alkohol oder ein Gemisch von beiden, eine Polyethylenglykolmonooleatverbindung, ein phenolischer Fettalkoholether oder ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist, wobei das Mittel zusätzlich SDS oder Glycerin enthält, wenn das Detergens nur ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist,
- (h) Einstellung des pH-Werts der IFN- β -Lösung auf einen Bereich von 3,5 bis 9,5,
- (i) Zugabe einer wirksamen Menge eines Füll-/Stabilisierungsmittels zur Lösung, und
- (j) Gefriertrocknen der Formulierung.
28. Verfahren zur Extraktion von unglykosyliertem rekombinantem Interferon- β (IFN- β) aus einem bakteriellen Wirt, der zu dessen Produktion transformiert wurde, und anschließende Reinigung und Formulierung des IFN- β , umfassend die folgenden Schritte:
- (a) Züchtung der transformierten Wirte in einem geeigneten Fermentationsmedium,
- (b) Konzentration des bakteriellen Wirts in dem Fermentationsmedium,
- (c) Aufbrechen der Zellwand und der Zellmembran des bakteriellen Wirts,
- (d) Entfernung von mehr als 99 Gew.-% der Salze aus dem aufgebrochenen Material durch Diafiltration oder Zentrifugation,
- (e) erneutes Aufbrechen des entsalzten aufgebrochenen Materials,
- (f) Zugabe eines Stoffes zu dem aufgebrochenen Material zur Erhöhung der Dichte oder Viskosität der Flüssigkeit oder zur Erzeugung eines Dichte- oder Viskositätsgradienten in der Flüssigkeit innerhalb des aufgebrochenen Materials,
- (g) Abtrennen des stark lichtbrechenden Materials von den Zelltrümmern durch Hochgeschwindigkeitszentrifugation,
- (h) Solubilisierung des stark lichtbrechenden Materials in einem wässrigen Puffer, der ein Reduktionsmittel enthält,
- (i) Extraktion des solubilisierten stark lichtbrechenden Materials mit 2-Butanol oder 2-Methyl-2-butanol,
- (j) Isolierung des stark lichtbrechenden Materials aus dem Extrakt,
- (k) Solubilisierung des resultierenden IFN- β -Teilchenniederschlags mit einer wässrigen Lösung von Natriumdodecylsulfat in einem Verhältnis von IFN- β zu Natriumdodecylsulfat von 1:3, wobei eine Lösung erhalten wird,
- (l) Einstellung des pH-Werts der Lösung auf 9,5 und Reduktion des solubilisierten IFN- β mit Dithiothreitol,
- (m) Reinigung des reduzierten IFN- β durch Chromatographie,
- (n) Oxidation des IFN- β aus Schritt (m),
- (o) weitere Reinigung des oxidierten IFN- β durch Gelchromatographie und Sammeln des Eluats, das das gereinigte IFN- β enthält,
- (p) Entsalzen des gereinigten IFN- β -Eluats an einer Entsalzersäule, die äquilibriert und eluiert wird mit Natriumlaurat bei einem pH-Wert von 9,0 bis 9,8,
- (q) Erniedrigung des pH-Werts des Eluats auf einen Wert von 3,0,
- (r) Zentrifugation und Filtration der Lösung zur Entfernung des Niederschlags,
- (s) Zugabe einer wirksamen Menge eines oder mehrerer nicht-ionischer biokompatibler polymerer Detergentien oder einer Kombination von einem oder mehreren der Detergentien und einem anderen Solubilisierungs- oder Stabilisierungsmittel zum Filtrat zur Solubilisierung und Stabilisierung des IFN- β , wobei das Detergens ein ethoxylierter Fettalkoholether und Laurylether, eine Octylphenoxypolyethoxyethanolverbindung, ein modifizierter oxyethylierter oder oxypropylierter geradkettiger Alkohol oder ein Gemisch von beiden, eine Polyethylenglykolmonooleatverbindung, ein phenolischer Fettalkoholether oder ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist, wobei das Mittel zusätzlich SDS oder Glycerin enthält, wenn das Detergens nur ein Blockcopolymer aus Propylenoxid und Ethylenoxid ist,
- (t) Einstellung des pH-Werts der Lösung auf 4,0 bis 9,5,
- (u) Zugabe eines geeigneten Füll-/Stabilisierungsmittels in einer Konzentration von 0,25 % bis 10 %, und
- (v) Gefriertrocknen der IFN- β -Lösung.

29. Verfahren nach Anspruch 28, wobei das Füll-/Stabilisierungsmittel von Schritt (u) Dextros ist und wobei der Gefriertrocknungsschritt (v) als ein Verfahren mit r Einschluß eines zweistufigen Anstiegs durchgeführt wird.

- 5 30. Verfahren nach Anspruch 29, wobei das Gefriertrocknungsverfahren die folgenden Schritte umfaßt:
- (a) Einfrieren der IFN- β -Lösung für etwa 2 bis etwa 6 Stunden bei einem Temperaturbereich von -70° bis -20° C,
 - (b) Gefriertrocknen der gefrorenen IFN- β -Lösung von Schritt (a) für 12 bis 48 Stunden,
 - (c) Erhöhen in einer Rate von 3° C/Stunde bis 12° C/Stunde auf einen Temperaturbereich von -5° bis $+10^{\circ}$ C,
 - 10 (d) Einhalten des Temperaturbereichs von Schritt (c) für 6 bis 18 Stunden,
 - (e) Erhöhen mit 3° C/Stunde bis 12° C/Stunde auf einen Temperaturbereich von $+10^{\circ}$ C bis $+25^{\circ}$ C und
 - (f) Einhalten des Temperaturbereichs von Schritt (e) für mindestens 12 Stunden.

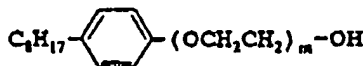
Revendications

1. Composition pharmaceutique stable d'une matière appropriée pour l'administration parentérale à des animaux ou des humains comprenant une quantité thérapeutiquement efficace d'une protéine interféron- β recombinée, non glycosylée dans un milieu excipient inerte à base aqueuse comprenant, comme solubilisant/stabilisant, une quantité efficace d'un ou de plusieurs détergents polymériques non ioniques biocompatibles ou solubilisants/stabilisants, dans laquelle le détergent est un éther d'alcool gras éthoxylé et le lauryléther, un composé octylphénoxypropyléthoxyéthanol; un alcool à chaîne linéaire modifié oxyéthylé ou oxypropylé ou un mélange des deux; un composé monooléate de polyéthylène-glycol; un éther d'alcool gras phénolique, ou un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène, dans laquelle la composition contient de plus, du SDS ou du glycérol lorsque le détergent est seulement un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène.
2. Composition suivant la revendication 1, dans laquelle le milieu excipient comprend de plus un agent solubilisant ou stabilisant supplémentaire.
3. Composition suivant la revendication 1 ou 2, qui est sous forme liquide ou lyophilisée.
4. Composition suivant la revendication 3, qui est sous forme liquide et dans laquelle l'agent solubilisant ou stabilisant supplémentaire est le glycérol dans un intervalle de concentration (v/v) de 5% à 50% et le détergent non ionique biocompatible se situe dans un intervalle de concentration (v/v) de 0,0005% à 5%.
5. Composition suivant l'une quelconque des revendications 1 à 4, dans laquelle le détergent est :
 - 40 (a) un mélange de composés d'éther d'alcool gras éthoxylé et de lauryléther ayant la formule :



dans laquelle n est une distribution de 1 à 30 et est centré sur $n = 12$ et w est une distribution de 9 à 17, contenant une partie significative de $w = 11$;

(b) un composé octylphénoxypropyléthoxyéthanol ayant la formule :



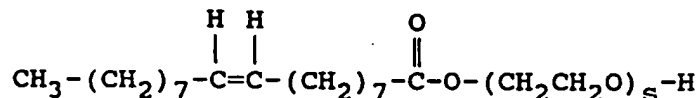
dans laquelle m est un entier entre 5 et 60;

(c) un mélange d'alcools à chaîne linéaire modifiés oxyéthylés ou oxypropylés ou les deux ayant la formule :



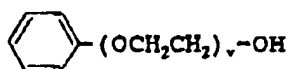
dans laquelle p est un entier entre 1 et 10, q est 0 ou un entier entre 1 et 10 et r est un entier entre 6 et 14;

(d) un composé monooléate de polyéthylèneglycol ayant la formule :



dans laquelle s est un entier entre 1 et 10;

(e) un éther d'alcool gras phénolique ayant la formule :



dans laquelle v est un entier entre 10 et 200, ou

(f) des copolymères séquencés d'oxyde de propylène et d'oxyde d'éthylène (polyols Pluronic®).

6. Composition suivant la revendication 5, dans laquelle le détergent est :

(a) un mélange de polyoxyéthylène-12-lauryléthers qui est un éther d'alcool gras éthoxylé, le polyoxyéthylène-12-lauryléther (Trycol® LAL-12); un polyoxyéthylène-12-laurylalcool (Macol® LA-12) ou un polyoxylène-12-laurylalcool (Siponic® L-12);

(b) un composé octylphénoxypropoxyéthanol dans lequel m est un entier entre 20 et 50;

(c) un mélange de composés alcool à chaîne linéaire modifié oxyéthylé ou oxypropylé ou les deux dans lequel :

p est un entier entre 1 et 5;

q est 0 ou un entier entre 1 et 5, et

r est un entier entre 10 et 12;

(d) un composé monooléate de polyéthylèneglycol dans lequel s est un entier entre 2 et 6;

(e) un éther d'alcool gras phénolique dans lequel v est un entier entre 50 et 150, ou

(f) un copolymère séquencé d'oxyde de propylène ou d'oxyde d'éthylène.

7. Composition suivant la revendication 6, dans laquelle le détergent est :

(a) un polyoxyéthylène-12-laurylalcool (Macol® LA-12);

(b) un composé octylphénoxypropoxyéthanol dans lequel m est 30 ou 40;

(c) un alcool à chaîne linéaire modifié oxyéthylé (Plurafac® C17);

(d) un éther de polyéthylèneglycol dans lequel s est 3, et

(e) un éther d'alcool gras phénolique dans lequel v est 100.

8. Composition suivant la revendication 7, dans laquelle le détergent est :

(a) un polyoxyéthylène-12-laurylalcool (Macol® LA-12);

(b) un composé octylphénoxypropoxyéthanol (Triton® X305);

(c) un alcool à chaîne linéaire modifié oxyéthylé (Plurafac® C17);

(d) un monooléate de polyéthylèneglycol 400 (Nopalcol® 4-0).

9. Composition suivant l'une quelconque des revendications 1 à 8, dans laquelle l'intervalle de concentration (v/v) du détergent non ionique est de 0,005% à 5%.

10. Composition suivant la revendication 3, qui est sous forme liquide et qui comprend de plus, un ou plusieurs agents stabilisants carbohydatés ou non carbohydatés dans un intervalle de concentration (v/v) de 0,025% à 10%.

11. Composition suivant la revendication 10, dans laquelle les agents stabilisants carbohydatés sont le sucrose, le fructose, le dextrose, le dextran, le mannitol, le sorbitol, l'inositol, le galactitol, le xylitol, le lactose ou la tréhalose et l'agent stabilisant non carbohydaté est la sérum-albumine humaine.

12. Composition suivant la revendication 3, qui est lyophilisée et susceptible d'être reconstituée et dans laquelle le milieu excipient inerte comprend de plus, un tampon et un agent de charge/stabilisant, dans laquelle le tampon se situe dans un intervalle de concentration de 1 à 50 mM et est de l'acétate ou du phosphate, et l'agent de charge/stabilisant est du dextrose seul dans un intervalle de concentration (v/v) de 1% à 10%; un mélange de dextrose dans un intervalle de concentration (v/v) de 0,1% à 5% avec du mannitol à une concentration (v/v) de 0,5% à 5%; un mélange de dextrose dans un intervalle de concentration (v/v) de 0,1% à 5% avec de la sérum-albumine humaine dans un intervalle de concentration (v/v) de 1% à 5% ou de la sérum-albumine humaine seule dans un intervalle de concentration (v/v) de 1% à 5%.
13. Composition suivant la revendication 12, dans laquelle le tampon est du phosphate à une concentration de 10 mM à 25 mM et l'agent de charge/stabilisant est du dextrose à une concentration (v/v) de 5% ou un mélange de dextrose et de mannitol dans un rapport de concentration de 1/5 à 1/20 de dextrose/mannitol.
14. Composition suivant la revendication 12 ou 13, dans laquelle le détergent non ionique est le polyoxyéthylène-12-lauryléter à une concentration (v/v) de 0,05% à 5%, l'agent de charge/stabilisant est un mélange de dextrose à une concentration (v/v) de 0,1% à 0,2% et de mannitol à une concentration (v/v) de 1% à 3%; le tampon est du phosphate dans un intervalle de concentration de 15 mM à 25 mM; l'IFN- β est à une concentration de 0,125 à 2 mg/ml et le pH de la composition se situe de 3,5 à environ 9,5.
15. Composition suivant la revendication 14, dans laquelle le détergent non ionique est un polyoxyéthylène-12-laurylalcool (Macol® LA-12) à une concentration (v/v) de 0,15%; l'agent de charge/stabilisant est un mélange de dextrose à une concentration (v/v) de 0,2% et de mannitol à une concentration (v/v) de 2%; le tampon phosphate est à une concentration de 20 mM; l'IFN- β est à une concentration de 0,25 mg/ml et le pH de la composition est de 6.
16. Composition suivant l'une quelconque des revendications 1 à 15, dans laquelle les détergents sont aux concentrations respectives (v/v) indiquées en pourcentages comme suit : 0,1% de polyoxyéthylène-12-lauryléter et 0,05% de monooléate de polyéthylèneglycol 400 (Nopalcol® 4-0); 0,1% d'octylphénoxy-polyéthoxyéthanol (Triton® x305); 0,05% de monooléate de polyéthylèneglycol 400 (Nopalcol® 4-0); 0,1% d'octylphénoxy-polyéthoxyéthanol (Triton® X405), et 0,05% de monooléate de polyéthylèneglycol 400 (Nopalcol® 4-0), et l'IFN- β est à une concentration de 0,25 mg/ml.
17. Composition suivant l'une quelconque des revendications 1 à 16, dans laquelle l'interféron- β recombiné est l'IFN- β_{ser17} .
18. Composition suivant l'une quelconque des revendications 1 à 17, dans laquelle l'IFN- β est maintenu en solution à un pH de 3,5 à 9,5 pendant une période de 24 heures à 4 °C.
19. Composition suivant la revendication 18, dans laquelle le pH est neutre.
20. Composition suivant la revendication 3, qui est lyophilisée et susceptible d'être reconstituée et dans laquelle le milieu excipient inerte comprend de plus, un tampon ou un agent de charge/stabilisant et un tampon; la concentration du tampon est entre 1 et 50 mM et est de l'acétate ou du phosphate, et l'agent de charge/stabilisant est un mélange de sorbitol entre 0,1% et 5% et de mannitol entre 0,1% et 5%.
21. Composition suivant la revendication 20, dans laquelle le détergent non ionique est un polyoxyéthylène-12-lauryléter à une concentration (v/v) entre 0,05% et 5%; le tampon est du phosphate à un intervalle de concentration entre 15 mM et 25 mM; l'IFN- β est à une concentration entre 0,125 et 2 mg/ml et le pH est entre 3,5 et 9,5.
22. Composition suivant la revendication 21, dans laquelle le détergent non ionique est du polyoxyéthylène-12-laurylalcool (Macol® LA-12) à une concentration (v/v) de 0,15%; le tampon phosphate est à une concentration de 20 mM; l'IFN- β est à une concentration de 0,25 mg/ml et le pH de la composition est 6.

23. Composition suivant la revendication 1, comprenant de plus un excipient pharmaceutiquement acceptable par lequel la composition est rendue administrable par voie orale.
24. Procédé de criblage pour un ou plusieurs détergents polymériques non ioniques biocompatibles ou pour un mélange d'un ou de plusieurs de ces détergents avec un autre agent solubilisant ou stabilisant capable de solubiliser et de stabiliser des compositions pharmaceutiques d'interféron- β (IFN- β) recombiné non glycosylé, comprenant les étapes de :
- (a) passage de l'IFN- β extrait, purifié dans du dodécylsulfate de sodium (SDS) sur une colonne de dessalement équilibrée par du laurate de sodium dans un tampon d'élution à pH 9,0-10,0 pour former un éluat;
 - (b) diminution du pH de l'éluat à environ 2-3,3;
 - (c) centrifugation et filtration de l'éluat pour éliminer le laurate de sodium précipité;
 - (d) addition au filtrat d'une quantité efficace d'un ou de plusieurs des détergents ou du mélange d'un ou de plusieurs des détergents avec un autre agent solubilisant ou stabilisant;
 - (e) ajustement du pH du filtrat à 3,5 à 9,5;
 - (f) repos du filtrat pendant environ 25 heures à pH 3,5 à 9,5, et
 - (g) détermination si l'IFN- β recombiné est maintenu en solution.
25. Procédé suivant la revendication 24, dans lequel l'autre agent solubilisant ou stabilisant est le glycérol dans un intervalle de concentration (v/v) d'environ 5% à environ 50%.
26. Procédé de préparation de compositions pharmaceutiques, stables de protéine interféron- β (IFN- β) recombinée non glycosylée comprenant les étapes de :
- (a) extraction de l'IFN- β d'organismes hôtes brisés, transformés pour produire la protéine;
 - (b) purification de l'IFN- β au moyen, comme dernière étape de purification, d'une étape de dessalement à un intervalle de pH de 8,5 à 10, en utilisant un tampon d'élution contenant un sel d'acide gras ayant une chaîne contenant de 10 à 12 carbones pour former une fraction dessalée;
 - (c) diminution du pH de la fraction dessalée à un pH de 2 à 4 pour précipiter le sel d'acide gras;
 - (d) élimination du sel précipité de la fraction par centrifugation et filtration;
 - (e) addition à la fraction dessalée d'une quantité efficace d'un ou de plusieurs détergents polymériques non ioniques biocompatibles ou d'un mélange d'un ou de plusieurs détergents polymériques non ioniques biocompatibles et d'un autre agent solubilisant ou stabilisant, en des quantités efficaces pour solubiliser et stabiliser l'IFN- β , où le détergent est un éther d'alcool gras éthoxylé et le lauryl éther, un composé octylphénoxypropyléthanol; un alcool à chaîne linéaire modifié oxyéthylé ou oxypropylé ou un mélange des deux; un composé monooléate de polyéthylèneglycol; un éther d'alcool gras phénolique, ou un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène où la composition contient de plus du SDS ou du glycérol lorsque le détergent est seulement un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène;
 - (f) ajustement du pH de la fraction dans un intervalle de 4,0 à 8,0;
 - (g) addition d'une quantité efficace d'un agent de charge/stabilisant à la fraction, et
 - (h) lyophilisation de la formulation.
27. Procédé de préparation de compositions pharmaceutiques, stables de protéine interféron- β (IFN- β) recombinée non glycosylée comprenant les étapes de :
- (a) isolement de corps réfractaires contenant l'IFN- β d'un organisme hôte transformé pour produire l'IFN- β ;
 - (b) solubilisation des corps réfractaires au moyen de laurate de sodium;
 - (c) extraction et purification de l'IFN- β à partir de la matière réfractaire solubilisée au moyen de laurate de sodium comme agent de solubilisation primaire;
 - (d) diminution du pH de l'IFN- β purifié à un pH de 2 à 4;
 - (e) centrifugation et filtration de la solution d'IFN- β purifié pour éliminer le laurate de sodium précipité pour créer une fraction d'IFN- β ;
 - (f) dessalement de la fraction d'IFN- β à un pH de 2 à 4;
 - (g) addition à la solution de protéine IFN- β d'une quantité efficace d'un ou de plusieurs détergents polymériques non ioniques biocompatibles ou d'un mélange d'un détergent polymérique non ionique biocompatible et d'un autre agent solubilisant ou stabilisant, pour solubiliser et stabiliser l'IFN- β , où le détergent est un éther d'alcool gras éthoxylé et le lauryl éther, un composé octylphénoxypropyléthanol; un alcool à chaîne linéaire modifié oxyéthylé ou oxypropylé ou un mélange des deux;

un composé monoooléate de polyéthylèneglycol; un éther d'alcool gras phénolique, ou un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène où la composition contient de plus du SDS ou du glycérol lorsque le détergent est seulement un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène;

5 (h) ajustement du pH de la solution d'IFN- β dans un intervalle de 3,5 à 9,5;

(i) addition à la solution d'une quantité efficace d'un agent de charge/stabilisant, et

(j) lyophilisation de la formulation.

28. Procédé d'extraction d'interféron- β (IFN- β) recombiné non glycosylé à partir d'un hôte bactérien transformé pour le produire et ensuite purification et formulation de l'IFN- β comprenant les étapes de :

(a) mise en croissance des hôtes transformés dans un milieu de fermentation approprié;

(b) concentration de l'hôte bactérien dans le milieu de fermentation;

(c) rupture des parois cellulaires et des membranes cellulaires de l'hôte bactérien;

(d) élimination de plus de 99% en poids des sels de la fraction de rupture par diafiltration ou centrifugation;

(e) nouvelle rupture de la fraction de rupture dessalée;

(f) addition d'une matière à la fraction de rupture pour augmenter la densité ou la viscosité de, ou pour créer un gradient de densité ou de viscosité dans, le liquide de la fraction de rupture;

(g) séparation de la matière réfractaire des débris cellulaires par centrifugation à vitesse élevée;

(h) solubilisation de la matière réfractaire dans un tampon aqueux comprenant un agent réducteur;

(i) extraction de la matière réfractaire solubilisée par du 2-butanol ou du 2-méthyl-2-butanol;

(j) isolement de la matière réfractaire du liquide d'extraction;

(k) solubilisation du culot particulaire d'IFN- β résultant avec une solution aqueuse de dodécylsulfate de sodium à un rapport IFN- β :dodécylsulfate de sodium de 1:3 pour former une solution;

(l) ajustement du pH de la solution à 9,5 et réduction de l'IFN- β solubilisé par du dithiothréitol;

(m) purification de l'IFN- β réduit par chromatographie;

(n) oxydation de l'IFN- β de l'étape (m);

(o) purification supplémentaire de l'IFN- β oxydé par chromatographie sur gel et récolte de l'éluat contenant l'IFN- β purifié;

(p) dessalement de l'éluat d'IFN- β purifié dans une colonne de dessalement équilibrée et éluée dans du laurate de sodium à pH 9,0 à 9,8;

(q) diminution du pH de l'éluat à 3,0;

(r) centrifugation et filtration de la solution pour éliminer le précipité;

(s) addition au filtrat d'une quantité efficace d'un ou de plusieurs détergents polymériques non ioniques biocompatibles ou d'un mélange d'un ou de plusieurs des détergents et d'un autre agent solubilisant ou stabilisant, pour solubiliser et stabiliser l'IFN- β , où le détergent est un éther d'alcool gras éthoxylé et le lauryléther, un composé octylphénoxypropyléthoxyéthanol; un alcool à chaîne linéaire modifié oxyéthylé ou oxypropylé ou un mélange des deux; un composé monoooléate de polyéthylèneglycol; un éther d'alcool gras phénolique, ou un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène où la composition contient de plus du SDS ou du glycérol lorsque le détergent est seulement un copolymère séquencé d'oxyde de propylène et d'oxyde d'éthylène;

(t) ajustement du pH de la solution de 4,0 à 9,5;

(u) addition d'un agent de charge/stabilisant approprié en une concentration de 0,25% à 10%, et

(v) lyophilisation de la solution d'IFN- β .

29. Procédé suivant la revendication 28, dans lequel l'agent de charge/stabilisant de l'étape (u) est du dextrose et dans lequel la lyophilisation de l'étape (v) est réalisée par un procédé incorporant une rampe en deux étapes.

30. Procédé suivant la revendication 29, dans lequel le procédé de lyophilisation comprend les étapes de :
(a) congélation de la solution d'IFN- β pendant environ 2 à environ 6 heures à une température dans un intervalle de -70 ° à -20 ° C;

(b) lyophilisation de la solution d'IFN- β gelée de l'étape (a) pendant 12 à 48 heures;

(c) rampe à une vitesse de 3 ° C/heure à 12 ° C/heure jusqu'à une température dans un intervalle de -5 ° à +10 ° C;

(d) maintien de l'intervalle de température de l'étape (c) pendant 6 à 18 heures;

(e) rampe de 3 ° C/heure à 12 ° C/heure jusqu'à une température dans un intervalle de +10 ° C à +25 ° C, et

EP 0 270 799 B1

(f) maintien de l'intervall de température de l'étape (e) pendant au moins 12 heures.

5

10

15

20

25

30

35

40

45

50

55

FIG. 1A

| | |
|-----------------------------------|---|
| Fermentation | |
| Cell concentration | |
| Cell wall and membrane disruption | homogenization |
| Diafiltration | 5 mM EDTA |
| Redisruption | 2 mM EDTA; 1% octanol (v/v); homogenization |
| Sucrose suspension | 15-23% sucrose (w/w) |
| Centrifugation | 10,000 - 15,000 xg |
| Paste solubilization | 1-2% sodium laurate, 20 mM phosphate buffer, 50 mM DTT, pH 9-10 (with sonication) |
| Reduction | 10 mM DTT; 1-2% sodium laurate; 2 mM EDTA; pH 9; heat to 50°C for 10 min. under nitrogen; cool to about 25°C |
| Sephacryl® S200 column | 10 mM Tris • HCl; pH 9.2; 1-2% sodium laurate; 1 mM EDTA |
| Oxidation | Iodosobenzoic acid (IBA) equimolar; protein:IBA; 1-2% sodium laurate; 2 mM sodium pyrophosphate; pH 9; 1 mM EDTA |
| Concentration | pH 9.0 |
| Sephacryl® S200 column | 10 mM Tris • HCl; pH 9.2; 0.1-0.5% sodium laurate; 1 mM EDTA |

FIG. 1B

| | |
|-------------------------------|---|
| Concentration | 10 mM Tris • HCl; pH 9.2; 0.1-0.5% sodium laurate, 1 mM EDTA |
| Sephadex® G-75 column | 10 mM Tris • HCl; pH 9.2; .1-.5% sodium laurate; 1 mM EDTA |
| Sephadex® G-25 column | 0.1% laurate; 10 mM Tris • HCl; pH 9.2 |
| pH Adjustment | pH of eluate lowered quickly to 3 with 1.0 N HCl; sodium laurate precipitates |
| Centrifugation and Filtration | To remove precipitated sodium laurate |
| Stabilization | 0.15% Trycol LAL-12 added |
| Neutralization | pH raised to between 6.0 and 7.2 |
| Polyol addition | 5% dextrose |
| Pre-filtration | 0.45 μ M |
| Sterile filtration | 0.22 μ M |
| Lyophilization (immediate) | |
| Final Container Product | |

FIG. 2A

| | |
|---------------------------------|--|
| Fermentation | |
| Cell concentration | |
| Cell membrane disruption | homogenization |
| Diafiltration | 5 mM EDTA |
| Redisruption | 2 mM EDTA; 1% octanol (v/v); homogenization |
| Sucrose suspension | 15-23% sucrose (w/w) |
| Centrifugation | 10,000 - 15,000 xg |
| Paste solubilization | 2% SDS phosphate buffered saline |
| Reduction | 10 mM DTT; 2% SDS; 2 mM EDTA; pH 9; heat to 50°C for 10 min. under nitrogen; cool to about 25°C; adjust pH to 7.4 with glacial acetic acid |
| Organic extraction | 2-butanol/suspension (v/v) |
| Acid precipitation | pH 6.2; 2 mM DTT; 0.1% SDS |
| Centrifugation | 10,000 - 15,000 xg |
| Acid precipitate solubilization | 2% SDS; 5 mM EDTA; 50 mM phosphate buffer |
| Reduction | 20 mM DTT; pH 8.5; heat to 50° for 10 min. under nitrogen; cool to about 25°C |
| Sephacryl® S200 column | 50 mM acetate; pH 5.5; 1% SDS; 1 mM EDTA |
| Oxidation | Iodosobenzoic acid (IBA) equimolar; protein:IBA; 0.1% SDS; 2 mM sodium pyrophosphate; pH 9; 1 mM EDTA |
| Concentration | pH 5.5 |
| Sephacryl® S200 column | 50 mM acetate; pH 5.5; 0.1% SDS; 1 mM EDTA |

FIG. 2B

Concentration

Sephadex® G-75 column

50 mM acetate; pH 5.5; 0.1% SDS;
1 mM EDTA

Sephadex® G-25 column

0.1% sodium laurate (transfer
component) in 10 mM Tris-HCl,
pH 9.2

pH Adjustment

pH of eluate lowered quickly
with 1.0 N HCl to pH 3; sodium
laurate precipitates

Centrifugation and Filtration

To remove the precipitated sodium
laurate

Stabilization

0.1% Plurafac C-17 added

Neutralization

pH raised to between 6.0 and 7.2

Polyol addition

5% dextrose

Pre-filtration

0.45 μ M

Sterile filtration

0.22 μ M

Lyophilization (immediate)

Final Container Product

FIG. 3
LINEAR NONISOTHERMAL STABILITY STUDY

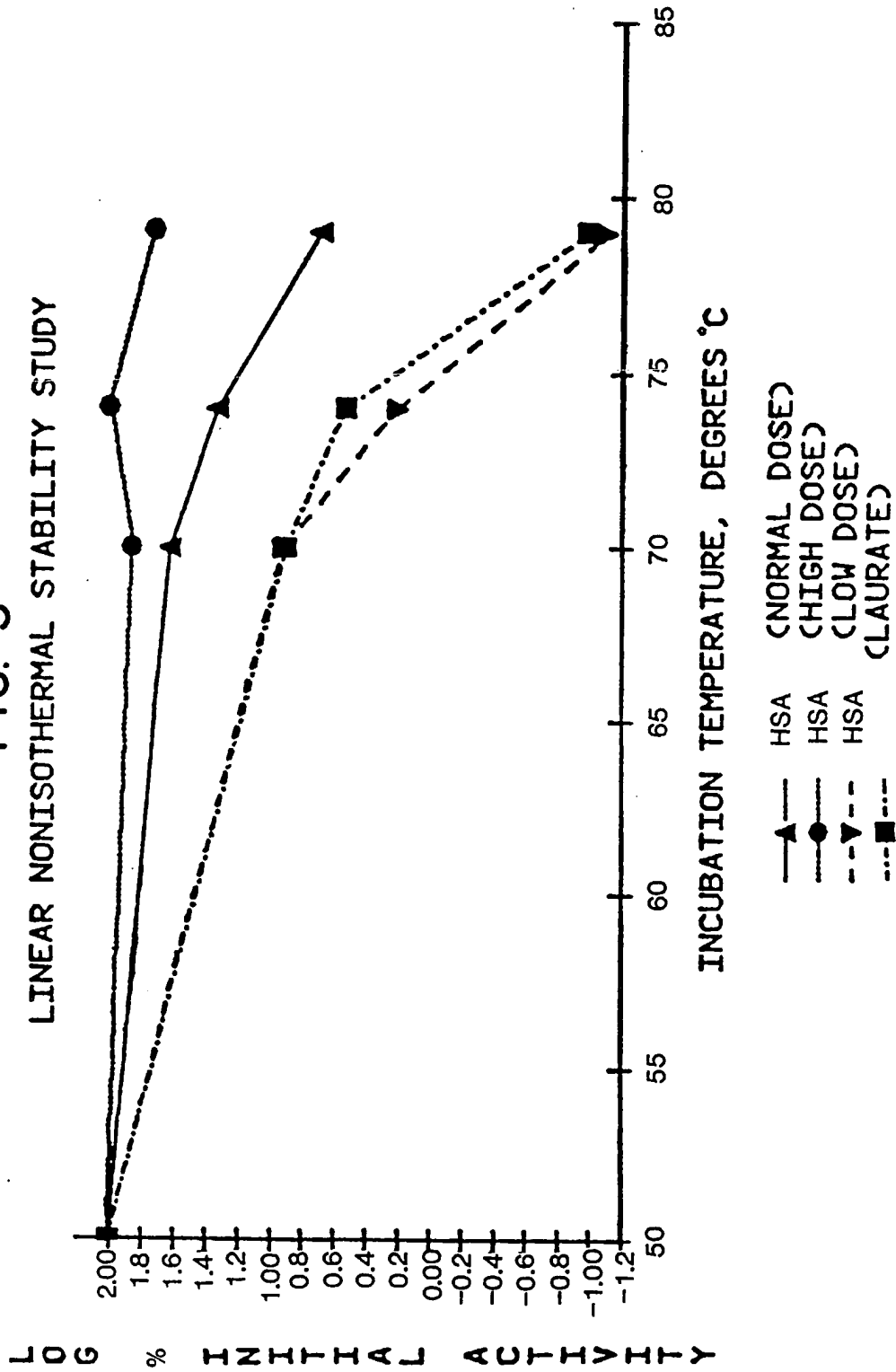


FIG. 4
LINEAR NONISOTHERMAL STABILITY STUDY

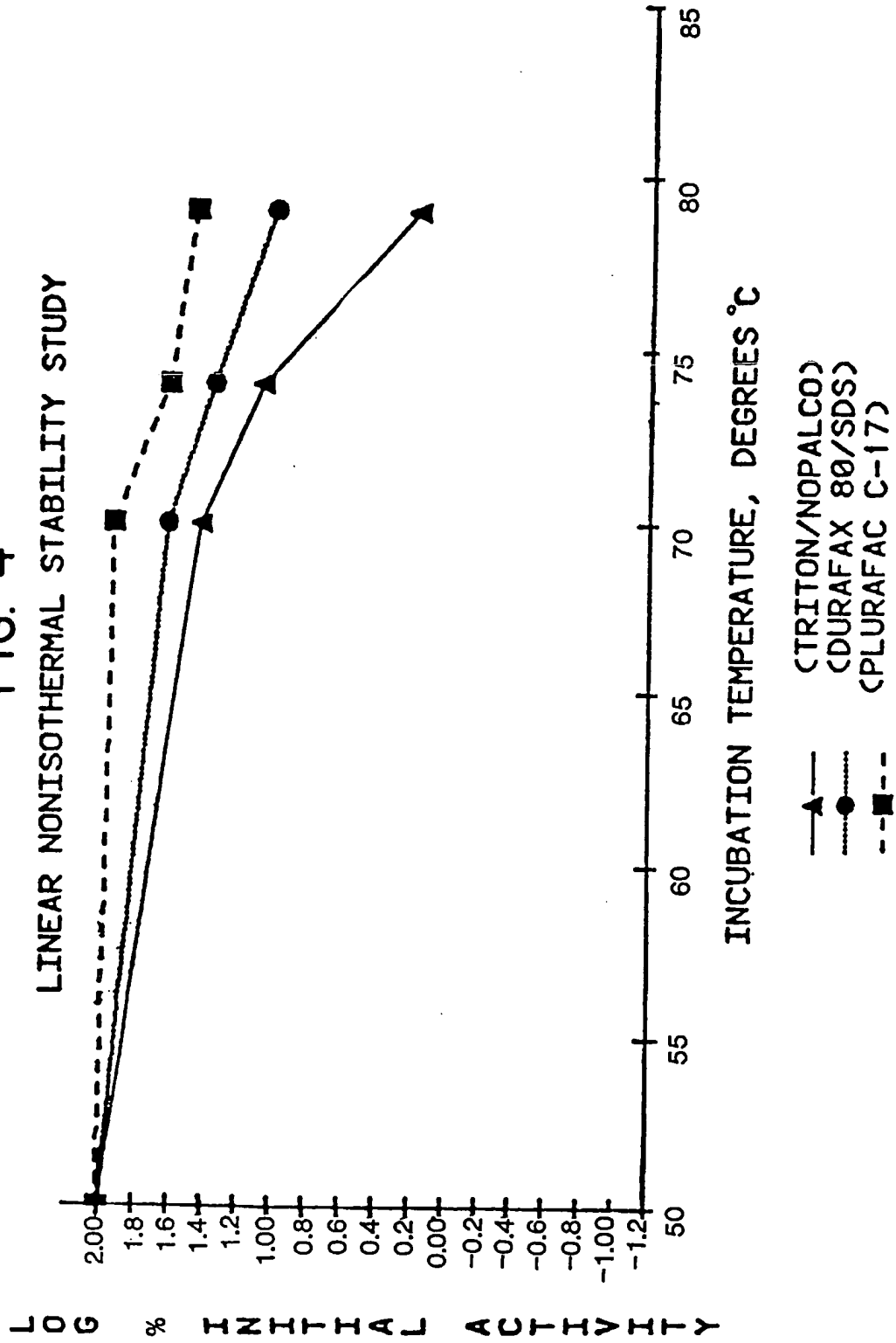


FIG. 5

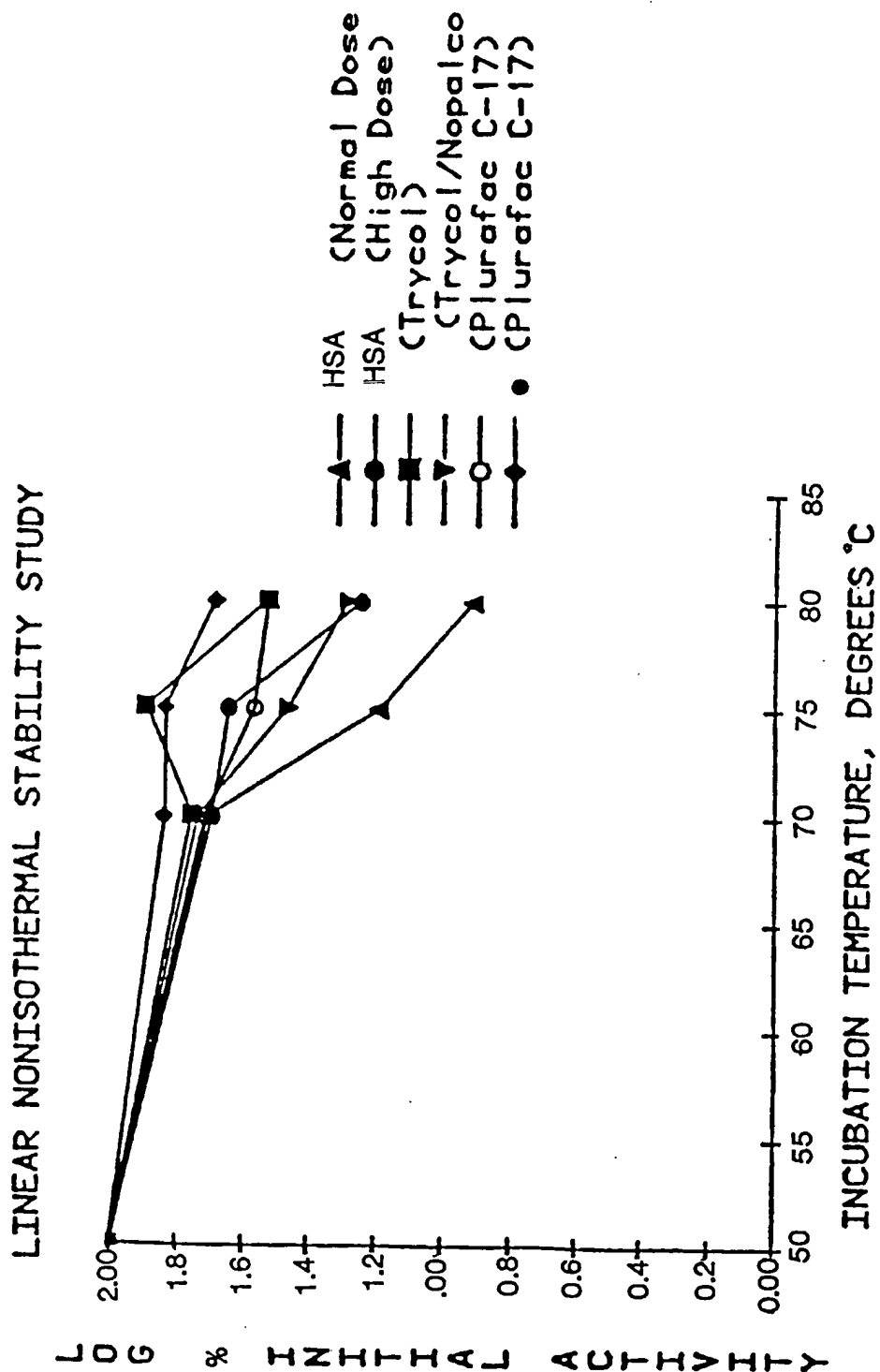


FIG. 6

LINEAR NONISOTHERMAL STABILITY STUDY

